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ESTUDO DA VARIABILIDADE GENÉTICA NAS PLAQUETAS: IMPLICAÇÕES FUNCIONAIS E CLÍNICAS

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Resumo

Os polimorfismos genéticos das proteínas plaquetárias podem induzir alterações funcionais, aumentando a suscetibilidade das plaquetas e a sua eventual participação em eventos potencialmente trombóticos. Uma vez que a hemóstase é o resultado do equilíbrio entre fatores protrombóticos e anti trombóticos, qualquer alteração genética com consequências funcionais pode alterar esse equilíbrio. Deste modo, diferenças na ativação e reatividade plaquetária entre diferentes indivíduos poderão influenciar a hemóstase normal e o aparecimento patológico de trombose.

A doença trombótica representa uma entidade complexa assente numa base multifatorial e poligénica. Apesar de já se conhecerem alguns fatores de risco hereditários relacionados com a trombose arterial, os mecanismos que contribuem para a sua fisiopatologia ainda não estão completamente compreendidos, embora estejam relacionados com o desenvolvimento da aterosclerose e a formação de um trombo rico em plaquetas no local de rutura da placa aterosclerótica. Fatores como a hiper-reactividade plaquetária também parecem conferir uma predisposição para o desenvolvimento de fenómenos trombóticos, embora os resultados dos diferentes estudos não sejam conclusivos. Incluído nos fenómenos trombóticos encontra-se o enfarte agudo do miocárdio (EAM) que representou, em 2015, 4,0% da totalidade de óbitos registados em Portugal, a terceira causa de morte dentro das doenças relacionadas com o aparelho circulatório. As plaquetas sanguíneas têm um papel fulcral na fisiopatologia do enfarte agudo do miocárdio, principalmente no enfarte agudo do miocárdio classificado como do tipo 1, ou seja, aquele em que houve rutura da placa aterosclerótica.

Neste trabalho estudámos as frequências alélicas e genotípicas de 10 polimorfismos (9 *SNP's* e 1 *VNTR*) pertencentes a 5 genes que codificam proteínas ligadas à fisiologia plaquetária (*GP1BA*, *ITGB3*, *ITGA2*, *P2RY12* e *NOS3*) numa população controlo, numa população de doentes que sofreram enfarte agudo do miocárdio antes dos 45 anos de idade e numa população de doentes que sofreram enfarte agudo do miocárdio depois dos 45 anos. Dos resultados obtidos podemos realçar que após este trabalho, a população

portuguesa encontra-se caracterizada no que respeita aos polimorfismos das principais proteínas plaquetárias, o que poderá servir como ferramenta importante de estudos futuros que avaliem o papel destas proteínas na predisposição individual a condições pró-trombóticas e na resposta à terapia anti trombótica. Em relação aos doentes foi encontrada uma associação entre 2 dos polimorfismos estudados e o enfarte agudo do miocárdio, nomeadamente o polimorfismo rs938043469 (807C>T) do gene *ITGA2*, nos doentes com mais de 45 anos e o polimorfismo rs2070744 (-786T>C) do gene *NOS3*, nos doentes com menos de 45 anos de idade.

Identificámos ainda a presença de codões sob seleção positiva na integrina $\alpha 2b\beta 3$, comparando a sequência nucleotídica de 10 mamíferos diferentes, incluindo o ser humano. Dos 7 codões identificados como estando a sofrer seleção positiva, 3 apresentavam polimorfismos no genoma humano.

Palavras-chave

Plaquetas sanguíneas; polimorfismos; enfarte agudo do miocárdio; seleção positiva.

Abstract

Genetic polymorphisms of platelet proteins may induce functional alterations, increasing the susceptibility of these platelets and their possible participation in potentially thrombotic events. Since hemostasis is the result of the balance between prothrombotic and antithrombotic factors, any genetic change with functional consequences may alter this balance. Thus, differences in platelet activation and reactivity between different individuals may influence normal hemostasis and the pathological onset of thrombosis.

Thrombotic disease represents a complex entity based on a multifactorial and polygenic basis. Although some hereditary risk factors related to arterial thrombosis are known, the mechanisms that contribute to its pathophysiology are not yet fully understood, although they are related to the development of atherosclerosis and the formation of a platelet-rich thrombus at the site of atherosclerotic plaque rupture. Factors such as platelet hyperreactivity also appear to confer a predisposition for the development of thrombotic phenomena, although the results of the different studies are not conclusive. Included in the thrombotic phenomena is acute myocardial infarction (AMI), which represented, in 2015, 4.0% of all deaths recorded in Portugal, the third cause of death within diseases related to the circulatory system. Blood platelets play a key role in the pathophysiology of acute myocardial infarction, especially acute myocardial infarction classified as type 1, that is, in which there was rupture of the atherosclerotic plaque.

In this work we studied the allelic and genotype frequencies of 10 polymorphisms (9 SNP's and 1 VNTR) belonging to 5 genes that encode proteins linked to platelet physiology (*GP1BA*, *ITGB3*, *ITGA2*, *P2RY12* and *NOS3*) in a control population, in a population of patients suffering from acute myocardial infarction before age 45, and in a population of patients who suffered acute myocardial infarction after age 45. From the results obtained, we can highlight that after this work, the Portuguese population is characterized with respect to polymorphisms of the main platelet proteins, which may serve as an important tool for future studies that evaluate the role of these proteins in the individual predisposition to pro- thrombotic conditions

and antithrombotic therapy. An association was found between 2 of the polymorphisms studied and acute myocardial infarction, namely the polymorphism rs938043469 (807C> T) of the gene ITGA2, in patients over 45 years old and polymorphism rs2070744 (-786T> C) of the NOS3 gene, in patients less than 45 years of age.

We also identified the presence of codons under positive selection in the α2bβ3 integrin, comparing the nucleotide sequence of 10 different mammals, including humans. Of the 7 codons identified as being undergoing positive selection, 3 had polymorphisms in the human genome.

Keywords

Blood platelets; polymorphisms; acute myocardial infarction; positive selection.

Organização da tese

A presente tese está organizada tendo por base quatro artigos originais em que o primeiro autor de cada artigo é o autor da tese. Destes quatro artigos, três estão publicados em revista indexada internacional e um está submetido para avaliação também em revista indexada internacional.

Os quatro artigos formam o corpo da tese, iniciando-se a tese com uma introdução geral ao tema e finalizando-se com uma discussão geral. Desta forma, a tese está dividida em cinco capítulos. No primeiro capítulo, "Introdução geral", faz-se uma síntese do papel das plaquetas na hemóstase, a importância dos recetores plaquetários na fisiologia normal da hemóstase e a sua participação nas mais variadas doenças tromboembólicas. Revê-se a etiologia do enfarte agudo do miocárdio e o papel das plaquetas nesse fenómeno. Destaca-se ainda o papel dos polimorfismos plaquetários nas doenças cardiovasculares e reveem-se os principais conceitos relacionados com a utilização de genómica comparativa na dentificação de codões a evoluir sob seleção positiva. Por fim apresentam-se os objetivos principais da tese. No segundo capítulo, "Frequências alélicas e genotípicas de polimorfismos plaquetários numa população portuguesa", que inclui um artigo já previamente publicado em revista indexada, descrevem-se as frequências alélicas e genotípicas de alguns dos polimorfismos estudados neste trabalho. No terceiro capítulo, "Papel dos polimorfismos plaquetários no enfarte agudo do miocárdio antes e depois dos 45 anos de idade", que inclui dois artigos, um previamente publicado e outro submetido para publicação, estuda-se o papel dos polimorfismos, anteriormente descritos na "Introdução geral", no enfarte agudo do miocárdio em duas populações subdivididas pela idade, antes e depois dos 45 anos. No quarto capítulo, "Seleção positiva na integrina $\alpha 2b\beta 3$", que inclui um artigo previamente publicado em revista indexada, identificam-se, em mamíferos, resíduos aminoacídicos da integrina $\alpha 2b\beta 3$ que evoluíram sob forte pressão seletiva. Finalmente, no quinto capítulo, "Discussão geral", discutem-se os principais resultados obtidos nos capítulos anteriores e apresentam-se linhas de investigação futuras. Após este último capítulo anexam-se três artigos previamente publicados em que o autor da

tese participou como coautor e em que foram usados resultados obtidos durante a execução prática e laboratorial do trabalho de investigação que originou a presente tese.

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Capítulo 1: Introdução geral

Plaquetas sanguíneas

As plaquetas são elementos figurados anucleados do sangue, de forma discoide biconvexa, com um diâmetro de 2 a 4 micrómetros (μm) e um volume de 7 a 8 fentolitros (fL). Têm origem a partir da fragmentação do citoplasma dos megacariócitos, grandes células poliploides da medula óssea. Cerca de 65% das plaquetas encontram-se em circulação, numa concentração de aproximadamente 250×10^9/L, estando as restantes armazenadas essencialmente no baço (Joseph E. Italiano and Hartwig 2007).

O principal papel fisiológico das plaquetas é a sua participação no processo hemostático, quer através da formação de agregados plaquetários que obliteram rapidamente a rutura de vasos sanguíneos, principalmente os de pequeno calibre, quer através da exposição de superfícies fosfolipídicas e libertação de substâncias que conduzem à ativação do sistema de coagulação. Também é reconhecida a sua importante participação noutros processos fisiopatológicos, dos quais se destacam os fenómenos tromboembólicos, que representam uma das principais causas de morte no mundo ocidental, a rejeição de transplantes, a metastização tumoral e a inflamação (Bergmeier and Wagner 2007, Goldschmidt et al. 2007, Nierodzik and Karpatkin 2007, Savage and Ruggeri 2007).

No caso de as plaquetas não serem utilizadas em processos como os anteriormente descritos, a sua vida média varia normalmente entre 5 e 9 dias. No fim desse período são removidas pelo sistema reticuloendotelial, nomeadamente no fígado (29,2%), no baço (37,2%) e na medula óssea (31,7%). A sua reposição, levada a cabo pelo tecido hematopoético, é realizada a uma taxa de 35×10^9/L/dia (Grozovsky et al. 2010).

Em condições normais, as plaquetas circulam sem aderir à parede dos vasos ou umas às outras, e sem se ligarem às moléculas adesivas presentes no plasma. Tal acontece devido à libertação constante, por parte do endotélio íntegro, de substâncias antiagregantes como a prostaciclina (prostaglandina I_2) e o óxido nítrico (NO) (Rex and Freedman 2007). No entanto, quando surge uma lesão vascular em que é destruída a integridade do endotélio, as

plaquetas aderem, através de recetores da membrana plaquetária, a determinados componentes sub-endoteliais. O mecanismo de adesão é complexo e envolve recetores plaquetários, substâncias plasmáticas e sub-endoteliais. Destas últimas, em que se incluem entre outras, a fibronectina, a trombospondina, a laminina e a vitronectina, é de destacar o colagénio, devido à sua importância no fenómeno de interação entre as plaquetas e o sub-endotélio vascular (Saad and Schoenberger 2018).

Em situações de alto *shear rate*, o principal recetor responsável pela adesão das plaquetas ao sub-endotélio é a glicoproteína (GP) Ib do complexo GPIb-IX-V (figura 1), constituída por duas subunidades ligadas por pontes dissulfúricas, a GPIbα e a GPIbβ (Andrews et al. 2007). A interação entre a GPIb e o colagénio necessita do fator de von Willebrand (vWF) que funciona como ponte de ligação. O vWF, que circula no plasma ligado não covalentemente ao fator VIII (FVIII) da cascata da coagulação, existe no plasma em séries de multímeros com pesos moleculares que variam entre 500 e 10 000 KDa, ligados por pontes dissulfúricas. É sintetizado pelos megacariócitos e pelas células endoteliais, sendo libertado por estas últimas, ou para o plasma ou para a matriz sub-endotelial. Este fator também poderá ser utilizado na ligação da GPIb a outras fibras da parede dos vasos sanguíneos e na interação plaqueta-plaqueta, ao interagir com outros recetores como a integrina αIIbβ3 (GPIIb-IIIa) (Andrews et al. 2007, Plow et al. 2007). O maior e mais externo constituinte da subunidade GPIbα, designado por glicocalicina, além de ser a porção responsável pela ligação ao vWF, também funciona como um dos recetores para a trombina da cascata da coagulação. A glicocalicina, fragmentada da estrutura do complexo GPIb-IX-V, circula normalmente no plasma em concentrações significativas. A sua medição é um indicador de variáveis como o número de plaquetas, a sua taxa de reposição e a presença de doenças caracterizadas por um número diminuído de plaquetas, como a trombocitopenia hipoplásica e a púrpura trombocitopénica idiopática (HadjKacem et al. 2016) (figura 1).

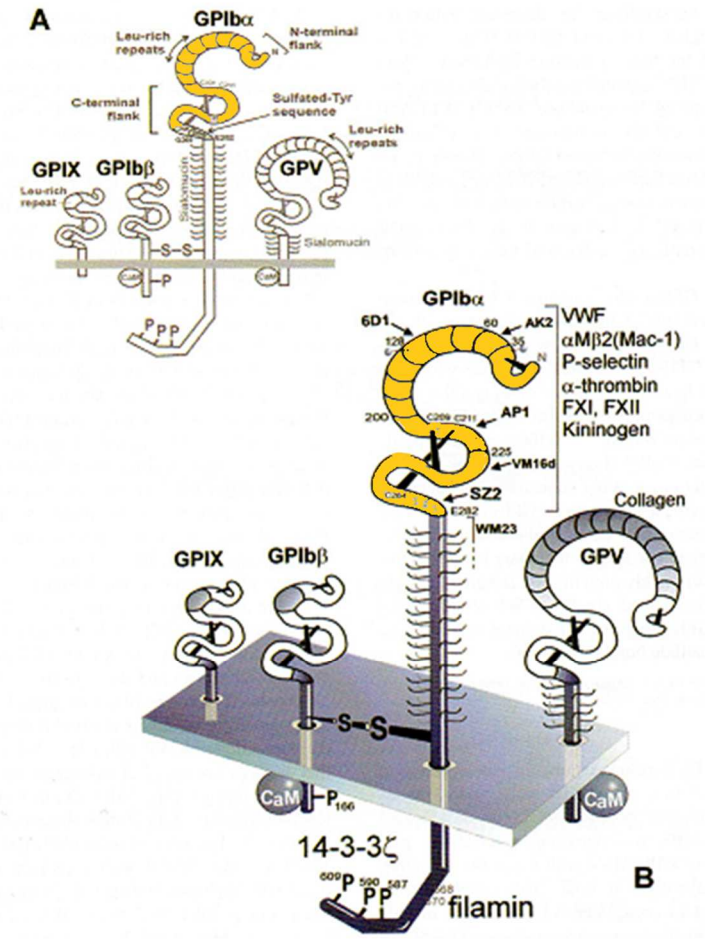

Figura 1. A) Domínios estruturais e **B)** domínios funcionais da GPIB-IX-V. A parte mais externa da subunidade GPIbα (glicocalicina), responsável pela ligação de vários agonistas ativadores da plaqueta como o vWF, MAC-1 (integrina αMβ2), P-selectina, trombina, FXI, FXII e cininogénio de alto peso molecular aparece realçado a amarelo, (adaptado de (Andrews et al. 2007)).

Entre outras glicoproteínas envolvidas no processo de adesão plaquetária ao sub-endotélio após a ligação da GPIb-IX-V ao vWF, destacam-se a integrina α2β1(GPIa-IIa) e a GPVI.

Recetores plaquetários

As plaquetas sanguíneas apresentam, quer na sua superfície externa, quer intracelularmente, uma quantidade considerável de recetores com

funções variadas. Devido à sua participação no fenómeno hemostático, as plaquetas que habitualmente circulam em vasos sanguíneos íntegros, ao passar em locais em que essa integridade se perdeu, participam na rápida reposição da integridade do vaso lesado. Recetores plaquetários como o recetor PGI_2 que se liga à prostaciclina (inibidor plaquetário) ou o recetor GPIb-IX-V com afinidade para o vWF (ativador plaquetário) são responsáveis por detetar os diferentes estímulos externos que normalmente sinalizam o estado do vaso sanguíneo e alterarem, se necessário, o estado da plaqueta sanguínea para responder de acordo com a situação em causa (Andrews et al. 2007, Clemetson and Clemetson 2007, Rex and Freedman 2007).

Apesar do elevado número de recetores conhecidos, esse número ainda pode vir a aumentar com os avanços tecnológicos desenvolvidos para a identificação de novas moléculas existentes na membrana plaquetária. Na tabela 1 estão sumariados os principais recetores implicados na participação das plaquetas nos eventos que ocorrem antes e durante uma lesão vascular.

Tabela I. Principais recetores plaquetários implicados na fisiologia plaquetária (Modesti et al. 1995, Andrews et al. 2007, Bahou 2007, Cattaneo 2007, Clemetson and Clemetson 2007, McEver 2007, Novinska et al. 2007, Plow et al. 2007, Rex and Freedman 2007).

Recetor	Principais ligandos	Família molecular	Cópias por plaqueta	Função
PGI_2	Prostaciclina	Sete domínios transmembranares	Desconhecido	Inibição
sGC	NO	Enzima intracelular	Desconhecido	Inibição
$P2Y_{12}$	ADP	Sete domínios transmembranares	≈425	Ativação
TXA_2/PGH_2	Tromboxano	Sete domínios transmembranares	≈2 000	Ativação
PAR1	Trombina	Sete domínios transmembranares	≈2 500	Ativação
GPIb-IX-V	vWF Trombina	LRR	≈50 000	Adesão e ativação
$\alpha 2\beta 1$	Colagénio	Integrinas	2 000 a 4 000	Adesão e ativação
GPVI	Colagénio	Superfamília Ig	≈3 000	Adesão e ativação
$\alpha IIb\beta 3$	Fibrinogénio vWF	Integrinas	50 000 a 80 000	Agregação e ativação
PECAM-1	PECAM-1	Superfamília Ig	≈7 500	Modulador da reatividade
P-selectina	Hidratos de carbono	Recetores de lectina do tipo C	≈10 000	Estabilizador de agregados plaquetários

PGI2 – prostaciclina; sGC – guanil-ciclase solúvel; NO – óxido nítrico; $P2Y_{12}$ – recetor purinérgico; ADP – adenosina difosfato; TXA_2/PGH_2 – tromboxano A_2/prostaglandina H_2; PAR1 – recetor ativado por protéases 1; GP – glicoproteína; vWF – fator de von Willebrand; LRR - Repetições ricas em leucina; Ig – imunoglobulinas; PECAM - molécula de adesão celular endotelial plaquetária.

Participação das plaquetas na hemóstase

O sistema hemostático depende de complexas interações entre a parede dos vasos, as plaquetas e os processos de coagulação e fibrinólise, envolvendo várias fases que incluem a hemóstase primária e secundária.

Uma hemóstase primária eficaz envolve três acontecimentos críticos: adesão, secreção e agregação plaquetária. Resumidamente, os principais passos deste processo podem ser enumerados da seguinte forma:

I. A adesão das plaquetas ao colagénio do sub-endotélio é, numa primeira fase, da responsabilidade do complexo GPIb-IX-V e do vWF. Após a exposição do sub-endotélio ao sangue circulante, o vWF plasmático tem tendência a desligar-se do FVIII e a ligar-se, principalmente, ao colagénio do sub-endotélio, aumentando naturalmente a densidade de moléculas de vWF naquele local específico. O vWF assim localizado facilita a interação entre si e a GPIb do complexo GPIb-IX-V, levando as plaquetas a aderirem ao local da lesão;

II. Numa segunda fase, a integrina $\alpha 2\beta 1$(GPIa-IIa) e a GPVI das plaquetas já aderidas interagem diretamente com o colagénio adjacente. Esta interação leva à ativação das plaquetas através de mecanismos de transdução do sinal (Brass et al. 2007);

III. Seguidamente, as plaquetas exibem uma série de modificações da sua forma, primeiro passando de discoides a esferoides e depois emitindo pseudópodes como resultado da ativação do sistema contráctil, dependente da polimerização da actina;

IV. Segregam, então, para o plasma, os conteúdos dos grânulos densos, dos grânulos α e, por vezes, dos lisossomas através de mecanismos de exocitose;

V. Algumas das substâncias libertadas, nomeadamente o ADP proveniente dos grânulos densos e o tromboxano A2 imediatamente sintetizado após a ativação da plaqueta, vão ativar plaquetas que se encontram nas proximidades, levando-as a expor à sua superfície grandes quantidades de recetores para o fibrinogénio (integrina αIIbβ3). O fibrinogénio servirá de ponte para as plaquetas agregarem quer entre si, quer às que já se encontram

aderidas ao sub-endotélio (Plow et al. 2007, Reed 2007, Savage and Ruggeri 2007). Forma-se, assim, um agregado plaquetário que oblitera a zona vascular lesada. Este fenómeno corresponde à primeira fase da hemóstase (hemóstase primária), e é responsável pela paragem das hemorragias resultantes da rutura de vasos, principalmente os de pequeno calibre (figura 2).

As plaquetas também intervêm na coagulação sanguínea (hemóstase secundária) e na fibrinólise. Assim, após a sua ativação, expõem superfícies pró-coagulantes, nomeadamente fosfolípidos como o fator plaquetário 3 (zonas ricas em fosfatidilserina), que participam na ativação da cascata da coagulação sanguínea e determinadas proteínas que formam complexos com os fatores ativados Va e Xa da coagulação (figura 2). As plaquetas ativadas expressam também locais de ligação para o cininogénio de alto peso molecular, para os fatores XIa e XIIIa, para a proteína C ativada e para o plasminogénio (Bouchard et al. 2007). Durante o processo de agregação, as plaquetas libertam ainda inibidores da fibrinólise, mais concretamente, a α2-antiplasmina e o inibidor tipo 1 do ativador do plasminogénio (PAI-1), os quais atrasam a destruição do coágulo pelas enzimas fibrinolíticas (Maron and Lozcalzo 2007).

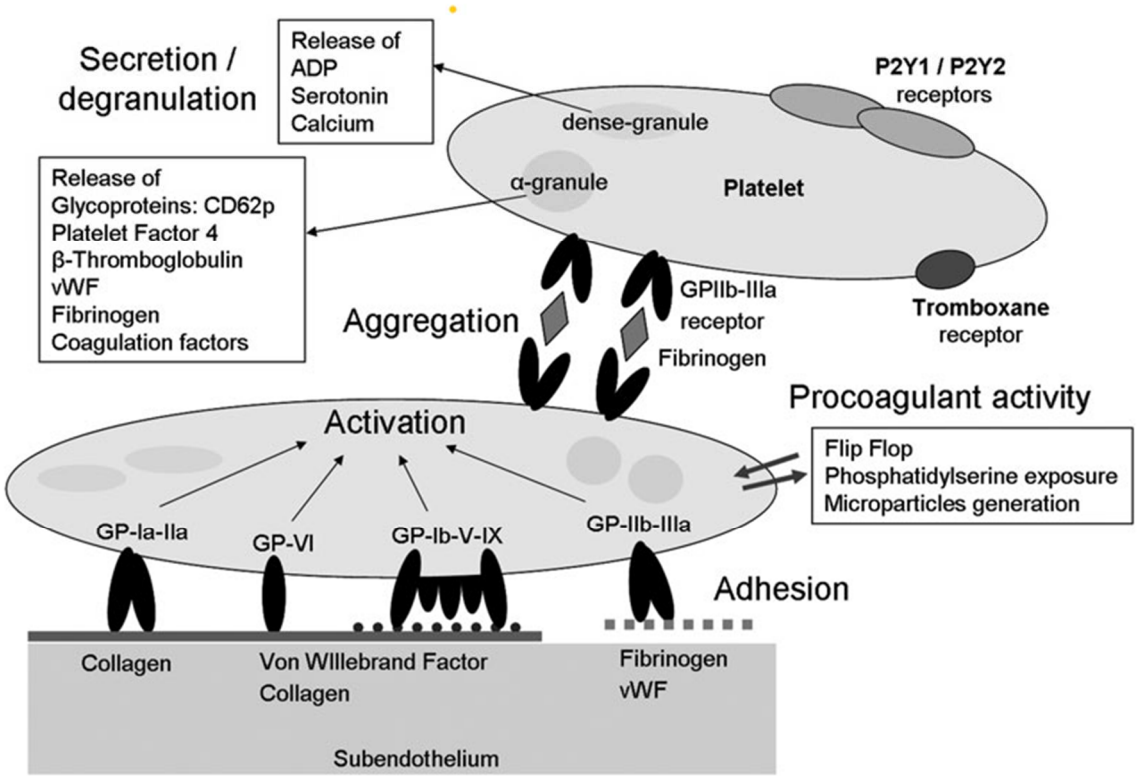

Figura 2. Resumo gráfico dos processos de adesão, ativação, secreção, agregação e atividade pró-coagulante das plaquetas sanguíneas após exposição do sub-endotélio ao sangue (adaptado de (Schoorl et al. 2013)).

Polimorfismos plaquetários

Pensa-se que fatores genéticos e ambientais estarão envolvidos na grande heterogeneidade funcional observada nas plaquetas, no entanto, os mecanismos subjacentes a esta heterogeneidade não estão completamente esclarecidos. Assim, se os polimorfismos genéticos das proteínas plaquetárias podem induzir alterações funcionais, poderão também aumentar a suscetibilidade dessas plaquetas e a sua eventual participação em eventos potencialmente trombóticos. Uma vez que a hemóstase é o resultado do equilíbrio entre fatores pró-trombóticos e anti-trombóticos, qualquer alteração genética com consequências funcionais pode alterar esse equilíbrio. Uma ativação plaquetária incontrolada (por hiper-reactividade basal e/ou rutura da placa aterosclerótica) poderá facilitar a formação de um agregado plaquetário

que, eventualmente, levará a disfunção trombótica. Deste modo, diferenças na ativação e reatividade plaquetária entre diferentes indivíduos poderão influenciar a hemóstase normal e o aparecimento patológico de trombose.

Embora não esteja completamente esclarecido qual o papel dos fatores genéticos na fisiopatologia e suscetibilidade à trombose arterial, assim como a sua relação com a eficácia das terapias anti-trombóticas comuns, sabemos que mais de 30% da variação natural da reatividade plaquetária parece estar relacionada com polimorfismos genéticos (Corral et al. 2004).

Como já foi anteriormente descrito, podemos considerar que alguns dos principais recetores plaquetárias envolvidos na hemóstase primária são:

- a GPIb-IX-V, responsável pela fase inicial de adesão das plaquetas ao sub-endotélio através do vWF;
- a integrina $\alpha 2\beta 1$ (GPIa-IIa) que se liga ao colagénio e estabiliza as plaquetas no local de adesão;
- o recetor $P2Y_{12}$ que responde ao ADP libertado pelas plaquetas previamente ativadas levando à ativação de novas plaquetas na zona da lesão vascular;
- a integrina $\alpha IIb\beta 3$ (GPIIb-IIIa), crucial para a ligação das plaquetas umas às outras durante a fase de agregação plaquetária através de pontes de fibrinogénio.

Atualmente estão descritos vários polimorfismos nos genes destes recetores. Muitos desses polimorfismos não apresentam significado clínico, ou pelo menos esse significado ainda não foi detetado. No entanto, várias dessas alterações polimórficas induzem modificações que têm influência na reatividade plaquetária, consoante o mecanismo afetado.

Um outro fator que intervém na função plaquetária é a presença do óxido nítrico (NO). O NO é sintetizado a partir da L-arginina por ação da enzima óxido nítrico sintetase endotelial (eNOS). Este gás difunde-se para as células adjacentes onde se liga ao grupo heme da guanil-ciclase solúvel (sGC) levando à formação de GMP cíclico, o qual induz o relaxamento das células do músculo liso do vaso e a inibição das plaquetas na corrente sanguínea conforme

anteriormente descrito. O GMP cíclico antagoniza os efeitos do cálcio livre determinando assim a inibição da função plaquetária.

Em relação à eNOS também estão descritos vários polimorfismos genéticos com um possível efeito na funcionalidade da enzima.

Na tabela 2 são apresentados os polimorfismos estudados neste trabalho referentes aos recetores plaquetários e à eNOS.

Tabela II. Proteínas plaquetárias e as alterações polimórficas estudadas nos respetivos genes.

Proteína	Gene	Polimorfismo
Subunidade GPIbα da GPIb-IX-V	*GP1BA*	rs2243093 (Kozak) rs6065 (HPA-2) VNTR
Subunidade α2 da integrina α2β1	*ITGA2*	rs938043469 (C807T)
$P2Y_{12}$	*P2RY12*	rs2046934 rs6801273 rs6798347
Subunidade β3 da integrina α2bβ3	*ITGB3*	rs5918 (PI^A)
eNOS	*NOS3*	rs2070744 (-786T>C) rs1799983 (894G>T)

Entre parêntesis indica-se o nome pelo qual o polimorfismo foi nomeado na altura da sua primeira descrição. VNTR – *variable number of tandem repeats*.

Papel das plaquetas sanguíneas na fisiopatologia do enfarte agudo do miocárdio

A doença trombótica representa uma entidade complexa assente numa base multifatorial e poligénica. Nas artérias os trombos desenvolvem-se após lesão da parede vascular ou rutura de uma placa de ateroma, o que em qualquer dos casos pode levar à ativação e agregação plaquetária. Nas veias a

formação dos trombos geralmente inicia-se com a geração de trombina em áreas de fluxo sanguíneo lento, sendo a fibrina o elemento predominante nestes casos. O tratamento dos estados pró-trombóticos também difere. Enquanto doentes com história de trombose arterial são tratados com anticoagulantes e antiagregantes, o risco de trombose venosa é tratado com anticoagulantes.

Vários estudos epidemiológicos têm descrito diferentes fatores de risco hereditários ou adquiridos que podem resultar em lesão endotelial ou alteração do equilíbrio hemostático (Sofi et al. 2005). Apesar de já se conhecerem alguns fatores de risco hereditários relacionados com a trombose arterial, como por exemplo a hiperhomocisteinemia ou níveis elevados de proteína C reativa (Cattaneo 1999, Thambidorai et al. 2004), os mecanismos que contribuem para a sua fisiopatologia ainda não estão completamente compreendidos, embora estejam relacionados com o desenvolvimento da aterosclerose e a formação de um trombo rico em plaquetas no local de rutura da placa aterosclerótica. Outros fatores, nomeadamente a hiper-reactividade das plaquetas sanguíneas, também parecem conferir uma predisposição para o desenvolvimento de fenómenos trombóticos, embora os resultados dos diferentes estudos não sejam conclusivos.

Entre os fenómenos trombóticos, o enfarte agudo do miocárdio (EAM) representou, em 2015, 4% da totalidade de óbitos registados em Portugal, a terceira causa de morte dentro das doenças relacionadas com o aparelho circulatório (Instituto nacional de estatística 2017).

As plaquetas sanguíneas têm um papel fulcral na fisiopatologia do EAM, principalmente no EAM classificado como do tipo 1, isto é, aquele em que houve rutura da placa aterosclerótica. Resumidamente, podemos descrever os eventos que levam ao EAM do tipo 1 da seguinte forma:

I. Rutura de uma placa aterosclerótica previamente presente numa artéria coronária;

II. Formação de agregados plaquetários que levam à oclusão dessa artéria;

III. Microembolização de vasos distais por agregados ateroscleróticos ricos em plaquetas;

IV. Vasoconstrição mediada por fatores libertados por plaquetas ativadas;

V. Reação inflamatória no miocárdio isquémico mediada por plaquetas ativadas (Gawaz 2004).

Desta forma, podemos concluir que a participação das plaquetas sanguíneas nestes eventos leva à oclusão da artéria estenosada e pode dificultar, numa fase posterior, a reperfusão e o retorno à normalidade do miocárdio isquémico.

Os sintomas principais do EAM incluem dor aguda no peito que pode irradiar para zonas adjacentes como o ombro, braço ou pescoço, dificuldade respiratória, sensação de desmaio, fraqueza e suores frios. Normalmente estes sintomas estão associados ao bloqueio completo de uma artéria coronária devido à rutura de uma placa aterosclerótica e consequente ativação plaquetária e formação de um trombo plaquetário. A isquemia do tecido miocárdico resultante leva ao aparecimento dos sintomas anteriormente descritos. Os critérios de diagnóstico de um EAM incluem a presença de sintomas de isquemia, a presença de biomarcadores no sangue como a elevação dos níveis de troponina cardíaca (cTn), alterações no padrão do eletrocardiograma (ECG) como a elevação do segmento ST (STEMI), a deteção através de testes de imagem (ecocardiografia ou ressonância magnética) de tecido necrosado ou com alterações funcionais de motilidade regionais, bem como a presença de trombose intra-coronária (angiografia coronária) (Thygesen et al. 2012, Vafaie 2016).

O tratamento antiplaquetário normalmente usado após a recuperação de um enfarte agudo do miocárdio é essencial para prevenir o risco aumentado de trombose. Atualmente recomenda-se o uso de terapia antiplaquetária dupla com ácido acetilsalicílico e um inibidor do recetor $P2Y_{12}$ (Valgimigli et al. 2018). Os inibidores do recetor $P2Y_{12}$ foram evoluindo ao longo dos últimos 20 anos e fármacos como o clopidogrel e o prasugrel (inibidores irreversíveis) ou o ticagrelor (inibidor reversível) são as opções farmacológicas atuais

recomendadas (Valgimigli et al. 2018). A escolha de qualquer um destes fármacos pode variar de acordo com o tipo de síndrome coronário agudo (STEMI vs. NSTEMI), tratamento a que o doente foi sujeito (*stent* vs. *bypass* coronário), risco de hemorragia ou presença de polimorfismos em enzimas metabolizadoras dos fármacos antiagregantes plaquetários, como o polimorfismo rs4244285 do citocromo P450 2C19 (Shuldiner et al. 2009).

Relação entre os polimorfismos plaquetários e as doenças cardiovasculares

Apesar da associação entre os polimorfismos plaquetários e determinados eventos tromboembólicos estar descrita na literatura em diferentes populações humanas, os resultados são contraditórios (Montagnana et al. 2014). O desenho experimental, diferenças na estrutura populacional e na apresentação clínica poderão interferir na interpretação dos resultados nos diferentes estudos.

Relativamente aos polimorfismos estudados no presente trabalho, os resultados encontrados na literatura também não são unânimes. Diversos autores encontraram uma associação entre as variantes genéticas da GPIbα e um aumento de eventos isquémicos (Baker et al. 2001, Mikkelsson et al. 2001), enquanto outros não encontraram essa associação (Carter et al. 1998). Relativamente ao recetor do fibrinogénio, integrina αIIbβ3, o alelo Pl^{A2} do gene *ITGB3* em homozigotia foi associado a uma maior reatividade plaquetária e a um incremento do risco de enfarte do miocárdio em doentes jovens (Michelson et al. 2000, Mikkelsson et al. 2000). Dentre os polimorfismos descritos para a integrina $\alpha2\beta1$, o C807T no exão 7 resulta numa maior densidade do recetor na membrana plaquetária e embora os resultados também neste caso não sejam conclusivos, diferentes estudos mostraram uma associação dos polimorfismos com um incremento do risco trombótico (Kunicki and Nugent 2002). Outros polimorfismos com relevância na função plaquetária encontram-se nos recetores do ADP. A ativação dependente do ADP é mediada por dois recetores designados por $P2Y_1$ e $P2Y_{12}$. A ligação do ADP ao recetor $P2Y_{12}$ é

essencial para a exposição da integrina αIIbβ3 na sua forma ativa e consequente agregação e estabilização do agregado plaquetário. São poucos os estudos realizados sobre este recetor, mas foram encontrados dois haplótipos diferentes, H1 e H2. Uma vez que os polimorfismos deste recetor podem influenciar a sua função, alguns estudos mostraram a importância da sua análise na avaliação do risco trombótico (Fontana et al. 2003, Cavallari et al. 2007). A associação entre os polimorfismos da eNOS e o risco de doenças cardiovasculares também tem sido extensamente estudada, embora, como nos casos anteriores, os resultados dos diferentes estudos não sejam totalmente concordantes (Colombo et al. 2003, Mathew et al. 2008).

Seleção positiva de aminoácidos pertencentes a recetores plaquetários

O rácio entre as alterações nucleotídicas que não levam a alterações aminoacídicas (substituições sinonímicas) e alterações nucleotídicas que implicam alterações aminoacídicas (substituições não-sinonímicas) calculada através da fórmula ω=dN/dS permite compreender como é que uma proteína está a evoluir ao longo do tempo. Muito simplisticamente, podemos dizer que se ω for igual a 1 não está a ocorrer qualquer tipo de seleção, se for menor que 1 temos seleção negativa, ou seja, as alterações nucleotídicas que alteram um aminoácido não serão favorecidas pela seleção natural, e se for maior que 1 temos seleção positiva, ou seja, as alterações nucleotídicas que originam alterações de aminoácidos serão favorecidas pela seleção natural. Quando analisada toda a proteína, o rácio dN/dS dificilmente será superior a 1, pois na proteína as regiões mais estruturais não permitem grande variação aminoacídica. No entanto, alguns codões por estarem mais expostos ou terem uma função muito específica, podem sofrer uma grande variabilidade aminoacídica. Por isso, nos últimos anos foram desenvolvidos métodos estatísticos que medem a probabilidade de um determinado codão estar a evoluir com um rácio dN/dS estatisticamente superior a 1.

A variação genética das proteínas plaquetárias exibida entre diferentes espécies pode revelar aspetos importantes relacionados com a função da

proteína ou de determinados elementos funcionais dentro dessa proteína. Pode também servir de indicador da interação entre a proteína e o meio externo, sendo que por meio externo devemos entender quer os outros componentes que fazem parte da constituição do organismo, quer elementos exógenos ao organismo, como microrganismos que eventualmente possam entrar em contacto com a proteína em questão. Como é sabido, genericamente podemos dizer que a interação entre um determinado organismo e o seu meio externo determina a evolução das suas estruturas de maneira a uma melhor adaptação às contingências do meio, levando a uma seleção dos indivíduos que apresentem, em termos globais, melhores características que lhes permitam sobreviver nessas condições. Em termos de seleção genética podemos dizer que as alterações nucleotídicas ocorrem ao acaso, e que se forem vantajosas podem fixar e aumentar as capacidades do indivíduo. Em relação aos recetores plaquetários, nomeadamente em relação à subunidade β3 da integrina α2bβ3, está descrita no homem uma relação do tipo hospedeiro-agente patogénico em que esta subunidade serve como recetor para a entrada na célula endotelial e nas plaquetas de um hantavírus específico (vírus dos Andes) que irá desencadear uma doença pulmonar potencialmente fatal (Matthys et al. 2010). No entanto, o vírus só consegue entrar na célula se o resíduo 33 for uma leucina (L). Se eventualmente este resíduo for substituído por uma prolina (P), o vírus já não consegue infetar as células endoteliais através da integrina $\alpha_v\beta$3 nem as plaquetas através da integrina α2bβ3 (Matthys et al. 2010). Este é um exemplo clássico em que uma simples alteração nucleotídica não sinonímica confere uma grande vantagem ao indivíduo. Como caso de estudo, podemos conjeturar que a variante 33P apareceu ao acaso na população e que poderá aumentar de frequência em zonas onde a infeção vírica provocada pelo hantavírus seja mais intensa. Curiosamente, nos humanos, este polimorfismo (rs5918) é um exemplo de substituição nucleotídica sinonímica muito bem estudada devido à sua participação na trombocitopenia neonatal aloimune (Mueller-Eckhardt et al. 1989).

Objetivos gerais e específicos do trabalho

Tendo em consideração a importância dos polimorfismos plaquetários na resposta das plaquetas, podemos colocar a hipótese que estas variações poderão eventualmente potenciar o desenvolvimento de doenças cardiovasculares como o enfarte agudo do miocárdio, influenciando mesmo o seu aparecimento precoce em doentes que exibam as variações genéticas descritas. A controvérsia à volta das implicações clínicas destas variações levanta a necessidade de estudos em populações específicas que nos permitam uma melhor compreensão do papel dos polimorfismos plaquetários.

Assim, o objetivo geral deste trabalho prende-se com o estudo de polimorfismos que exercem uma influência na função plaquetária e o seu possível envolvimento nas doenças trombóticas, nomeadamente no enfarte agudo do miocárdio.

Para conseguir este objetivo, elaborou-se o trabalho descrito nos capítulos 2, 3 e 4 desta tese, onde serão focados os seguintes objetivos específicos:

I. Descrição das frequências alélicas e genotípicas dos polimorfismos de recetores plaquetários e da eNOS na população portuguesa;

II. A análise das frequências alélicas e genotípicas dos polimorfismos em doentes que sofreram enfarte agudo do miocárdio subdivididos em dois grupos: doentes com mais e doentes com menos de 45 anos;

III. Comparação das frequências obtidas entre as várias populações estudadas e a sua importância clínica;

IV. Estudo evolutivo de recetores plaquetários, usando a comparação entre 10 sequências nucleotídicas de mamíferos referentes à integrina $\alpha 2b\beta 3$, um recetor fulcral para o desempenho das plaquetas durante a agregação, com o intuito de estudar possíveis variações devido a pressão seletiva.

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Capítulo 2: Frequências alélicas e genotípicas de polimorfismos plaquetários numa população portuguesa

Artigo 1

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Allelic and genotypic frequencies of platelet glycoprotein polymorphisms in a Portuguese population.

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Abstract

Introduction and objectives: We studied genotypic and allelic frequencies of polymorphisms that can affect platelet function, namely the Kozak, VNTR and HPA-2 polymorphisms of glycoprotein Ibα, the PlA polymorphism of glycoprotein IIIa and the C807T polymorphism of glycoprotein Ia, in a Portuguese population composed of 227 donors. Methods: PCR-RFLP was used to assess the Kozak, HPA-2, PlA and C807T polymorphisms. The VNTR polymorphism was discriminated by different weight bands on electrophoresis. Results: All genotypic frequencies were in Hardy-Weinberg equilibrium and do not differ from other Caucasian populations. Genotypic frequencies were 68.3%, 26.9% and 4.8% for PlA1/A1, PlA1/A2 and PlA2/A2 genotypes of the PlA polymorphism, 79.3%, 20.3% and 0.4% for TT, TC and CC genotypes of the Kozak polymorphism, 81.1%, 18.9% and 0.0% for aa, ab and bb genotypes of the HPA-2 polymorphism, 15.4%, 0.9%, 70.5%, 11.5%, 1.3% and 0.4% for BC, BD, CC, CD, DD and CE genotypes of the VNTR polymorphism, and 39.7%, 50.2% and 10.1% for CC, CT and TT

genotypes of the C807T polymorphism. Conclusions: The Portuguese population has now been characterized in terms of major platelet glycoprotein polymorphisms, which will be an important tool for further studies to assess the role of platelet glycoproteins in individual predisposition to prothrombotic conditions and response to antithrombotic therapy.

Resumo

Introdução e objetivos: Neste estudo determinámos as frequências alélicas e genotípicas de 5 polimorfismos que poderão afetar a funcionalidade plaquetária, nomeadamente os polimorfismos Kozak, VNTR e HPA-2 da glicoproteína Ibα, o polimorfismo PlA da glicoproteína IIIa e o polimorfismo C807T da glicoproteína Ia numa população portuguesa constituída por 227 dadores voluntários. Métodos: A técnica de PCR-RFLP foi usada para determinar os polimorfismos Kozak, HPA-2, PlA e C807T. O polimorfismo VNTR foi discriminado através do padrão eletroforético criado por bandas de peso molecular diferente. Resultados: As frequências genotípicas encontram-se dentro do equilíbrio de *Hardy-Weinberg* e não diferem estatisticamente de outras populações caucasianas. As frequências genotípicas encontradas foram 68,3, 26,9 e 4,8% para os genótipos PlA1/A1, PlA1/A2 e PlA2/A2 do polimorfismo PlA, 79,3, 20,3 e 0,4% para os genótipos TT, TC e CC do polimorfismo Kozak, 81,1, 18,9 e 0,0% para os genótipos aa, ab e bb do polimorfismo HPA-2, 15,4, 0,9, 70,5, 11,5, 1,3 e 0,4% para os genótipos BC, BD, CC, CD, DD e CE do polimorfismo VNTR, e 39,7, 50,2 e 10,1% para os genótipos CC, CT e TT do polimorfismo C807T. Conclusões: A população portuguesa encontra-se, assim, caracterizada no que respeita aos polimorfismos das principais glicoproteínas plaquetárias, o que poderá servir como ferramenta importante de estudos futuros que avaliem o papel das glicoproteínas plaquetárias na predisposição individual a condições pró-trombóticas e resposta à terapia anti trombótica.

Keywords

Platelet glycoprotein polymorphisms; Glycoprotein Ib-IX-V; Glycoprotein Ia-IIa; Glycoprotein IIb-IIIa.

Introduction

The process of hemostasis mediated by blood platelets is complex and involves several receptor-ligand interactions. Most platelet receptors are protein complexes with two or more polypeptide subunits in the platelet membrane. Interindividual differences in platelet responsiveness are usually found, and thus it is reasonable to suggest that in certain circumstances inherited variations in platelet glycoproteins (GP) may contribute to their functional heterogeneity. In fact, three major platelet membrane adhesion receptors, the GPIb-IX-V complex, integrin $\alpha 2\beta 1$(GPIa-IIa) and integrin $\alpha 2b\beta 3$ (GPIIb-IIIa), present genetic polymorphisms that can affect platelet responsiveness (Mueller-Eckhardt et al. 1989, Lopez et al. 1992, Murata et al. 1992, Kaski et al. 1996, Kritzik et al. 1998, Corral et al. 1999). Identification of such polymorphisms may thus be useful to assess disease predisposition and response to therapy.

The GPIb-IX-V complex mediates the initial adhesion of platelets to the subendothelial matrix under high shear stress conditions, via von Willebrand factor (vWF) binding (Kroll et al. 1996). The GPIb subunit is composed of two disulfide-linked polypeptides, GPIbα and GPIbβ. GPIbα, the largest protein of the complex, contains the binding sites for vWF and α-thrombin, both platelet activator ligands (Berndt et al. 1985). At least three previously described polymorphisms may influence the function and expression of the GPIbα subunit: a molecular weight polymorphism due to a variable number of tandem repeats (VNTR) within the mucin-like macroglycopeptide region of GPIbα (Lopez et al. 1992), a threonine-to-methionine substitution at amino acid 145 that forms the basis of the HPA-2 platelet alloantigen system (Murata et al. 1992), and a polymorphic variation at position -5 from the ATG start codon,

where either T or C is present, called the Kozak polymorphism (Kaski et al. 1996).

Integrin α2β1, also called the GPIa-IIa complex, is a major collagen receptor in platelets and other cell types. GPIa-IIa mediates platelet adhesion to collagen after an initial subendothelial interaction mediated by GPIb-IX-V and vWF (Staatz et al. 1989). The T allele of the C807T polymorphism within the coding region of the GPIa gene (I*TGA2*) has been associated with high expression of the receptor and with possible platelet hyperreactivity, even though this polymorphism does not alter amino acids (Kritzik et al. 1998, Corral et al. 1999).

The β3 integrin subunit (GPIIIa) plays a pivotal role in platelet aggregation. The β3 subunit forms a heterodimeric complex with the α2b integrin subunit (GPIIb). The major ligands for this glycoprotein are fibrinogen and vWF, when they are immobilized or in circulation after platelet activation (Savage et al. 2001). There are at least nine GPIIIa polymorphisms (Robinson et al. 2013), incompatibility for the HPA-1 (PlA) alloantigens being the most common cause of fetal and neonatal alloimmune thrombocytopenia in Caucasians (Mueller-Eckhardt et al. 1989). Studies on the functional consequences of this polymorphism in thrombotic diseases have yielded conflicting results (Goodall et al. 1999, Meiklejohn et al. 1999), but an increase in platelet reactivity has been reported in Pl (A2) carriers compared with Pl (A1/A1) individuals (Vijayan et al. 2005).

In view of the importance of genetic variants in platelet function and the controversy surrounding the clinical correlation between them and prothrombotic conditions, further studies appear to be required in specific populations, that will lead to a better understanding of molecular markers with important roles in platelet function. Since there are no data regarding platelet polymorphism frequencies in the Portuguese population, the aim of this study is to assess allelic and genotypic frequencies of polymorphisms that can affect platelet function, namely the Kozak, VNTR and HPA-2 polymorphisms of the *GP1BA* gene (GPIbα), the PlA polymorphism of the *ITGB3* gene (GPIIIa), and the

C807T polymorphism of the *ITGA2* gene (GPIa) in a normal Portuguese population.

Methods

Population

The study population consisted of 227 donors (56 male and 171 female) with ages ranging from 15 to 69 years (mean 27 years). This sample is part of a university population (students and employees) in which most students are female. At the time of the sample collection donors had no clinical signs of any hemorrhagic or thrombotic disease. Clinical data regarding blood pressure, presence of diabetes, body mass index, smoking status, physical exercise and family history of cardiovascular disorders were collected but were not used to exclude any donor. Every donor was born in Portuguese territory, as were their parents. All donors gave written informed consent to the protocol, which was approved by the local ethics committee.

Genotype analysis

Genomic DNA was extracted from peripheral blood cells collected by venipuncture in EDTA tubes using a PureLink™ Genomic DNA Mini Kit from Invitrogen. A region containing each polymorphism was amplified by PCR using 1 μg of DNA and 1 μM of specific primers (Table I). Except for the VNTR polymorphism, amplicons were then digested with specific restriction enzymes (Table I) and the digested fragments were visualized in a 2% ethidium bromide agarose gel.

Statistical analysis

Genotypic and allelic frequencies were calculated, and Hardy-Weinberg equilibrium was tested for the five polymorphisms by means of a chi-square test using observed vs. expected genotypic frequencies for a significance level of 0.05.

Table I. Primers and restriction enzymes used for genotypic discrimination.

Protein	Polymorphism	Primers	Restriction enzymes
GPIIIa	PI^A	F-GGACTTCTCTTTGGGCTCCT R-CTGTCTCCAGAGCCCTTGTC	*MspII*
GPIbα	Kozak	F-AGGGGGATCCACTCAAGG R-AGGCGAGTGTAAGGCATCAG	*BsuRI*
	HPA-2	F-GCCAGCCACCTAGAAGTGAA R-AAAAGCAAAAGGCAGGAGGT	*Lwel*
	VNTR	F-CTGGAGCCCACTCCAAGC R-TTGTGGCAGACACCAGGAT	-
GPIa	C807T	F-CTACCGGCCCATGTCTAAAT R-TCTTTGTCTTTTCCTTACTTTTTCA	*Hpy188I*

Results

Table II shows the allelic and genotypic frequencies obtained in the Portuguese population for the five polymorphisms. No major differences were observed compared with results from other Caucasian populations (Afshar-Kharghan et al. 2007). Concerning GPIbα, no bb genotype for the HPA-2 polymorphism or A allele for VNTR were found. The A allele has been described mainly in Japanese and Native American populations (Aramaki and Reiner 1999). Our population is in Hardy-Weinberg equilibrium for the five polymorphisms.

Table II. Genotypic and allelic frequencies of the five studied polymorphisms.

Protein	Polymorphism	Genotypes	Genotypic frequencies *n* (%)	Alleles	Allelic frequencies (%)
GPIIIa	PI^{A}	$PI^{A1/A1}$ $PI^{A1/A2}$ $PI^{A2/A2}$	155 (68.3) 61 (26.9) 11 (4.8)	PI^{A1} PI^{A2}	81.7 18.3
GPIbα	Kozak	TT CT CC	180 (79.3) 46 (20.3) 1 (0.4)	T C	89.5 10.5
	HPA-2	aa ab bb	184 (81.1) 43 (18.9) 0 (0.0)	a b	90.6 9.4
	VNTR	BC BD CC CD DD CE	35 (15.4) 2 (0.9) 160 (70.5) 26 (11.5) 3 (1.3) 1 (0.4)	B C D E	8.1 84.2 7.5 0.2
GPIa	C807T	CC CT TT	90 (39.7) 114 (50.2) 23 (10.1)	C T	64.8 35.2

Discussion

There were no available data regarding the allelic frequencies of platelet glycoprotein polymorphisms in the Portuguese population. All these polymorphisms can affect platelet function. GPIIb-IIIa is the most frequent glycoprotein in the platelet surface membrane and the Leu33Pro substitution (PlA polymorphism) is located in the PSI (plexin-semaphorin-integrin) domain, which is known to bind proteins and participate in integrin activation (Zang and Springer 2001). The HPA-2 polymorphism of GPIbα is near the binding site for vWF (Murata et al. 1992), VNTR affects GPIbα molecular size (Lopez et al.

1992) and Kozak is near the start codon, which could influence the expression rate of the gene (Kaski et al. 1996). The T allele of the C807T polymorphism of GPIa has also been associated with a higher expression of this glycoprotein on the platelet surface (Kritzik et al. 1998).

Platelet membrane glycoproteins, as key elements for normal primary hemostasis, have also been associated with platelet hyperreactivity and thrombotic mechanisms. Several clinical studies have reported conflicting data on the association of platelet polymorphisms and increased tendency for thrombosis, and differences between populations may contribute to the contradictory results (Carter et al. 1998, Baker et al. 2001, Mikkelsson et al. 2001). The Portuguese population has now been characterized in terms of major platelet glycoprotein polymorphisms. Our results are of interest in future studies that aim to assess the role of platelet glycoproteins as genetic markers involved in individual predisposition to prothrombotic conditions and in the patient response to therapy.

Ethical disclosures

Confidentiality of data

The authors declare that they have followed the protocols of their work center on the publication of patient data and that all the patients included in the study received sufficient information and gave their written informed consent to participate in the study.

Conflicts of interest

The authors have no conflicts of interest to declare.

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Capítulo 3: Papel dos polimorfismos plaquetários no enfarte agudo do miocárdio antes e depois dos 45 anos de idade

Artigo 1

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Acute myocardial infarction before and after 45 years: possible role of platelet receptors polymorphisms.

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Abstract

Introduction: We examined the potential role of platelet gene expression polymorphisms *GP1BA* (rs2243093, rs6065 and VNTR p.Ser415_Thr428(0_4)), *ITGB3* (rs5918), *ITGA2* (rs938043469) and *P2RY12* (rs2046934, rs6801273 and rs6798347) as risk factors for the development of acute myocardial infarction (AMI). Material and methods: The studied population was divided in 3 groups: Control (n=235), AMI≤45 years (n=44) and AMI>45 years (n=78). Control group was further divided in two subgroups (Control ≤45 and >45), and subgroups including only men were also considered for statistical analysis. Polymorphisms were detected by PCR (polymerase chain reaction) and restriction fragment length polymorphisms (RFLP) analysis. Results: Regarding non-genetic risk factors, the Control group differed statistically from AMI group≤45 years (p <0,05) in terms of smoking habits, hypertension, diabetes mellitus and obesity, and differed statistically from the AMI group>45 years (p <0.05) in the variables hypertension, diabetes mellitus, obesity, family history of thrombosis and high cholesterol. For the studied *ITGA2* polymorphism, a statistical difference was found when AMI>45 was compared with Control group, with a higher risk of AMI in TT genotype

(OR 2,852; CI 95% from 1,092 to 7,451; *p*=0,032). In *GP1BA* rs6065 polymorphism, a statistical difference, between Control≤45 only men and AMI≤45 only men, was found, with a higher risk in C/T genotype. (OR 5,568; CI 95% from 1,421 to 21,822; *p*=0,016), despite the low *n* included. The other polymorphisms studied did not show any statistical significant correlation. Conclusion: There is a statistically association between genotype T/T from *ITGA2* rs938043469 polymorphism and increased risk for AMI>45.

Resumo

Introdução: Determinou-se o papel de polimorfismos dos genes de expressão plaquetária *GP1BA* (rs2243093, rs6065 e o VNTR p.Ser415_Thr428(0_4)), *ITGB3* (rs5918), *ITGA2* (rs938043469) e *P2RY12* (rs2046934, rs6801273 e rs6798347) como fatores de risco para o enfarte agudo do miocárdio (EAM). Material e métodos: A amostra foi dividida em 3 grupos: Controlo (n=235), EAM≤45 anos (n=44) e EAM>45 anos (n=78). O grupo Controlo foi ainda dividido em dois subgrupos (Controlo ≤45 e >45). Subgrupos incluindo somente homens também foram considerados para fins estatísticos. Os polimorfismos foram estudados através de PCR e RFLP. Resultados: Em relação aos fatores de risco não-genéticos, o grupo Controlo diferia estatisticamente do grupo EAM≤45 anos (*p*<0,05) em termos de hábitos tabágicos, hipertensão, diabetes e obesidade, e também diferia do grupo EAM>45 anos (*p*<0,05) nas variáveis hipertensão, diabetes, obesidade, antecedentes familiares de trombose e colesterol. Para o polimorfismo estudado do gene *ITGA2*, verificou-se uma diferença estatisticamente significativa quando se compararam os grupos EAM>45 anos e Controlo, associando-se o genótipo TT a aumento de risco de EAM (OR 2,852; IC 95% de 1,092 a 7,451; *p*=0,032). No polimorfismo rs6065 do gene *GP1BA* foi encontrada uma diferença estatística quando comparados os grupos Controlo≤45 só homens e EAM≤45 só homens, associando-se o genótipo C/T a um maior risco de EAM (OR 5,568; IC 95% de 1,421 a 21,822; *p*=0,016), apesar do baixo *n*. Os outros polimorfismos não apresentaram nenhuma correlação significativa. Conclusão: Existe uma associação estatística

significativa entre o genótipo T/T do polimorfismo rs938043469 do gene *ITGA2* e o risco de EAM>45.

Keywords

Myocardial infarction and platelet polymorphisms, GP Ib-V-IX, GP Ia-IIa, GP IIb-IIIa, ADP P2Y12 receptor.

Introduction

Platelet receptors are critical for physiological platelet responses. They contribute for platelet thrombus formation after a vascular injury to the extent that they are responsible for platelet adhesion to damaged vessel walls and platelet aggregation. Four platelet receptors, namely GP Ib-IX-V, integrin $\alpha 2\beta 1$ (GP Ia-IIa), integrin $\alpha 2b\beta 3$ (GP IIb-IIIa) and P2Y12 ADP receptor are crucial for the development of normal hemostasis (Andrews et al. 2007, Cattaneo 2007, Clemetson and Clemetson 2007, Plow et al. 2007).

GP Ib-IX-V is responsible for initiate adhesion of platelets to subendothelial von Willebrand factor (vWF) under high shear stress conditions (Kroll et al. 1996). GP Ib subunit is composed of two disulfide-linked polypeptides, GPIb platelet alpha subunit and GPIb platelet beta subunit. GPIb platelet alpha subunit contains the binding sites for vWF and α-thrombin, both platelet activator ligands (Berndt et al. 1985) and is encoded by *GP1BA* gene (Andrews et al. 2007).

Integrin $\alpha 2\beta 1$ is a specific receptor for collagen present in platelets and other cell types that mediates platelet adhesion to collagen after prior platelet activation (Staatz et al. 1989). It is composed of two subunits, integrin subunit alpha 2 and integrin subunit beta 1, that are encoded by *ITGA2* and *ITGB1* genes, respectively (Clemetson and Clemetson 2007).

Integrin $\alpha 2b\beta 3$ plays a pivotal role in platelet aggregation. The major ligands for this glycoprotein are fibrinogen and vWF and interaction between these ligands and integrin $\alpha 2b\beta 3$ begins platelet plug formation (Savage et al. 2001). Integrin subunit alpha 2b and integrin subunit beta 3 are encoded by *ITGA2B* and *ITGB3* genes, respectively (Plow et al. 2007).

P2Y12 purinergic receptor is a Gi-coupled seven-membrane-spanning protein that interacts with ADP (Cattaneo 2007). ADP stimulate P2Y12-mediated inhibition of adenylyl cyclase and activates intracellular signal transduction pathways responsible for platelet aggregation stabilization (Kauffenstein et al. 2004, Cattaneo 2007). It is of clinical relevance the fact that this receptor is the target point for prasugrel and clopidogrel, both used as P2Y12 inhibitors in antiplatelet therapy for patients that presented acute coronary syndromes (Wiviott et al. 2007). This purinergic receptor is encoded by *P2RY12* gene (Cattaneo 2007).

These four platelet membrane receptors present genetic polymorphisms that can affect platelet responsiveness.

Three polymorphisms that can affect the function and expression of GP Ib-IX-V have been already described, namely a VNTR (variable number of tandem repeats), rs6065 (formerly, HPA-2) and rs2243093 (Kozak) polymorphisms. All of them lie in GP Ib platelet alpha subunit. VNTR is a molecular weight polymorphism within the mucin-like macroglycoprotein region of GP Ib platelet alpha subunit, specifically, a 13-amino-acid sequence repeat, where allele A gives rise to four repeats, B to three repeats, C to two repeats, D to one repeat and E lacks the 13-amino acid sequence (Lopez et al. 1992). Polymorphism rs6065 is a threonine to methionine substitution at amino acid 161 that forms the basis of the HPA-2 platelet alloantigen system (Murata et al. 1992). rs2243093 polymorphism is a variation at position -5 from the ATG start codon, where either T or C is present (Afshar-Kharghan et al. 1999).

Allele T of the polymorphism rs938043469 (C807T) within the coding region of integrin subunit alpha 2 of integrin $\alpha 2\beta 1$ (GPIa-IIa) has been associated with high expression of the receptor with possible platelet hyper reactivity despite being a polymorphism which do not alter amino acids (Kritzik et al. 1998, Corral et al. 1999).

There are at least nine polymorphisms described in integrin subunit beta 3 from integrin $\alpha 2b\beta 3$. Incompatibility for the HPA-1 (Pl^A) alloantigens is the most common cause of fetal and neonatal alloimune thrombocytopenia in Caucasians (Mueller-Eckhardt et al. 1989). This polymorphism (rs5918) is a

T>C transition creating a missense Leu59Pro variant. Studies regarding the functional consequences of this polymorphism in thrombotic diseases have yielded conflicting results (Goodall et al. 1999, Meiklejohn et al. 1999), but an increase in platelet reactivity was reported in C allele carriers (Pl^{A2}) compared with TT genotype individuals ($Pl^{A1/A1}$) (Vijayan et al. 2005).

A several number of single nucleotide polymorphisms (SNP's) in intron regions of *P2RY12* gene were already described, some of them associated with greater ADP-induced platelet aggregation such as rs2046934 (C>T), rs6801273 (T>C) and rs6798347 (G>A) (Fontana et al. 2003).

As platelets have an important role in the pathophysiology of acute myocardial infarction (Gawaz 2004) and controversy regarding the presence of platelet polymorphisms and increased cardiovascular risk still exists (Montagnana et al. 2014), further studies in specific populations are necessary to clarify the status of this relationship. In this study, we evaluated the potential role of 8 polymorphisms, one VNTR and 7 SNPs, of 4 genes codifying platelet receptors, *GP1BA*, *ITGB3*, *ITGA2* and *P2RY12*, as risk factors for the development of acute myocardial infarction. For that, two groups of patients who suffered acute myocardial infarction (AMI) were considered according to their ages: one with less than 45 years and another with more than 45 years.

Material and methods

Population

The studied population included only Portuguese Caucasians and donors were divided in 3 groups designated as Control, AMI≤45 years and AMI>45 years. Control group consisted of 235 donors (57 males and 178 females) with ages ranging from 15 to 69 years old (mean 27 years). Control group characterization was described previously (Pina-Cabral et al. 2013), except for the polymorphisms of ADP receptor gene *P2RY12*. At the time of the sample collection donors had no clinical signs of any hemorrhagic or thrombotic disease. AMI≤45 group consisted of 44 donors (40 males and 4 females) that suffered an acute myocardial infarction at or before 45 years,

with ages ranging from 27 to 45 years old (mean 42 years). AMI>45 group consisted of 78 donors (67 males and 11 females) that suffered an acute myocardial infarction after 45 years, with ages ranging from 51 to 97 years old (mean 67 years). Clinical data regarding smoking status, blood pressure, presence of diabetes, body mass index, family history of cardiovascular disorders and total cholesterol levels were collected for the 3 groups. All donors gave written informed consent to the protocol, which was approved by the local ethics committee.

Genotype analysis

Genomic DNA was extracted from peripheral blood cells collected by venipuncture in EDTA tubes using a PureLink™ Genomic DNA Mini Kit (Invitrogen). A region containing each polymorphism was amplified by PCR using 1μg of DNA and 1μM of specific primers (Table I). Except for the VNTR polymorphism, amplicons were then digested with specific restriction enzymes (Table I) and the digested fragments were visualized in a 2% ethidium bromide agarose gel.

Table I. Primers and restriction enzymes used for genotypic discrimination.

Platelet Receptor	Gene	Polymorphism	Primers	Restriction enzymes
α2bβ3	*ITGB3*	rs5918 (PI^A)	F-GGACTTCTCTTTGGGCTCCT R-CTGTCTCCAGAGCCCTTGTC	*MspII*
Ib-V-IX	*GP1BA*	rs2243093 (Kozak)	F-AGGGGGATCCACTCAAGG R-AGGCGAGTGTAAGGCATCAG	*BsuRI*
		rs6065 (HPA-2)	F-GCCAGCCACCTAGAAGTGAA R-AAAAGCAAAAGGCAGGAGGT	*Lwel*
		VNTR	F-CTGGAGCCCACTCCAAGC R-TTGTGGCAGACACCAGGAT	-
α2β1	*ITGA2*	rs938043469 (C807T)	F-CTACCGGCCCATGTCTAAAT R-TCTTTGTCTTTTCCTTACTTTTTCA	*Hpy188I*
P2Y12	*P2RY12*	rs2046934	F-TGCTGAAAATTGAAGCCATAC R-CAAAACAGGGCATACTTTCCA	*HpyCH4IV*
		rs6801273	F-TTGTTGAAATATCAGAAAATGTGAG R-AGTCCACCTGCTGCTATTGA	*Bsh1236I*
		rs6798347	F-TGATGTAAGTGGGGAAAGGAA R-CAAGTTTCAAACCCGAGGAA	*BseGI*

Statistical analysis

Genotypic and allelic frequencies for the Control group were calculated and Hardy-Weinberg equilibrium was tested for the eight polymorphisms by means of a chi-square test using observed vs. expected genotypic frequencies. Genotypic frequencies from AMI>45 and AMI≤45 groups were compared with the observed genotypic frequencies obtained from Control group using a chi-

square test. For those chi-square tests that presented a *p* value ≤0,05, odds ratios (OR) with 95% confidence intervals (CI) were calculated as estimates of the development of acute myocardial infarction as a function of the studied polymorphisms. Binary logistic regression analysis was performed to determine the association between AMI and the studied variables through calculation of OR and their 95% CI. Adjusted OR was estimated for the genetic and the physiological profile, using Multinomial logistic regression (adjusted maximum-likelihood estimation). Enter method was selected to enter independent variables into the analysis. IBM SPSS Statistics for Windows, Version 24.0 software was used for all statistical calculations.

Results

Table II shows the clinical data for the three studied groups. As can be seen in table II, the three groups studied showed statistically significant differences for non-genetic variables. Between control group and AMI≤45, smoking status (OR=5,651; CI 95% 2,2472-14,2041; *p*<0,001), hypertension (OR=16,755; CI 95% 4,521-62,500; *p*<0,001), diabetes mellitus (OR=60,948; CI 95% 3,644-1019,274; *p*=0,004) and obesity (OR= 5,6515; CI 95% 2,2472-14,2041; *p*<0,001) revealed significant differences. Between control group and AMI>45 group, hypertension (OR=58,823; CI 95% 17,544-200,000; *p*<0,001), diabetes mellitus (OR=80,119; CI 95% 4,812-1334,049; *p*=0,002), obesity (OR=3,472; CI 95% 1,013-11,905; *p*=0,048), family history of thrombosis (OR=8,707; CI 95% 2,657-28,532; *p*<0,001) and elevated cholesterol (OR=3,891; CI 95% 1,292-11,765; *p*<0,016) were the significantly different variables. Also, there is a significant heterogeneity in gender distribution among the studied groups (*p*<0,001 for both AMI groups), with a predominant number of males in both groups of patients.

Table II. Clinical characteristics of studied subjects.

	Control	AMI≤45	AMI>45
Number of subjects	235	44	78
Gender (male), n (%)	57 (24,3)	40 (90,9)*	67 (85,9)*
Active smokers, n (%)	62 (26,3)	30 (68,2)*	27(34,6)
Hypertension, n (%)	9 (3,8)	16 (36,4)*	54 (69,2)*
Diabetes mellitus, n (%)	0 (0,0)	10 (22,7)*	22 (28,2)*
Obesity (BMI above 30 kg/m^2), n (%)	8 (3,4)	11 (25,0)*	19 (24,4)*
Family history of thrombosis, n (%)	107 (45,5)	25 (56,8)	6 (7,7)*
Total cholesterol above 200 mg/dL, n (%)	59 (25,1)	11 (25,0)	43 (55,1)*

AMI – acute myocardial infarction. BMI – body mass index. Chi-square test was used to compare variables among control and AMI groups. * p value <0,05 when compared to Control group.

Genotypes were in Hardy-Weinberg equilibrium for all polymorphisms in the Control group. Table III shows the genotypic frequencies for the eight polymorphisms in the 3 groups studied and the chi-square *p* value obtained when AMI groups were compared with Control group for each polymorphism. Table III shows, also, the same comparison between groups but joining the homozygotic and heterozygotic genotypes for the allele considered of higher risk. This association of genotypes was performed due to the reduced numbers of patients that were found in some genotypes. As can be seen in table III, the chi-square *p* values for rs5918 polymorphism of integrin subunit beta 3 were not significant, which means that there are no statistical differences between Control and AMI groups (p=0,579 and p=0,359 for AMI>45 and p=0,407 and p=0,722 for AMI≤45).

Table III. Genotypic frequencies for rs5918 (PlA), rs2243093 (Kozak), rs6065 (HPA-2), VNTR, rs938043469 (C807T), P2Y12rs2046934, P2Y12rs6801273 and P2Y12rs6798347 polymorphisms.

Polymorphism genotypes	Control, n (%)	AMI>45, n (%)	*p* value[1]	AMI≤45, n (%)	*p* value[2]
rs5918 (T/T)	144 (69,2)	57 (74,0)		28 (66,7)	
rs5918 (T/C)	55 (26,4)	18 (23,4)	0,579	14 (33,3)	0,407
rs5918 (C/C)	9 (4,4)	2 (2,6)		0 (0,0)	
rs5918 (T/C+C/C)	64 (30,8)	20 (26,0)	0,359	14 (33,3)	0,722
rs2243093 T/T	168 (78,9)	62 (80,5)		29 (69,0)	
rs2243093 T/C	44 (20,7)	15 (19,5)	0,778	13 (31,0)	0,104
rs2243093 C/C	1 (0,4)	0 (0,0)		0 (0,0)	
rs2243093 T/C+C/C	45 (21,1)	15 (19,5)	0,728	13 (31,0)	0,118
rs6065 C/C	172 (81,5)	62 (80,5)		31 (73,8)	
rs6065 C/T	39 (18,5)	15 (19,5)	0,825	11 (26,2)	0,199
rs6065 T/T	0 (0,0)	0 (0,0)		0 (0,0)	
rs6065 C/T+T/T	39 (18,5)	15 (19,5)	0,825	11 (26,2)	0,199
VNTR B/C	35 (16,1)	12 (15,9)		11 (27,0)	
VNTR B/D	2 (0,9)	1 (1,1)		0 (0,0)	
VNTR C/C	153 (70,5)	49 (65,9)	0,373	27 (62,2)	0,236
VNTR C/D	24 (11,1)	14 (15,9)		4 (10,8)	
VNTR C/E	1 (0,5)	0 (0,0)		0 (0,0)	
VNTR D/D	2 (0,9)	1 (1,1)		0 (0,0)	
rs938043469 C/C	82 (38,7)	22 (28,2)		20 (45,5)	
rs938043469 C/T	109 (51,4)	41 (52,6)	0,011*	20 (45,5)	0,654
rs938043469 T/T	21 (9,9)	15 (19,2)		4 (9,0)	
rs938043469 C/T+T/T	130 (61,3)	56 (71,8)	0,057	24 (54,5)	0,358
rs2046934 A/A	177 (75,3)	44 (64,7)		31 (72,1)	
rs2046934 A/G	52 (22,1)	22 (32,4)	0,118	9 (20,9)	0,197
rs2046934 G/G	6 (2,6)	2 (2,9)		3 (7,0)	
rs2046934 A/G+G/G	58 (24,7)	24 (35,3)	0,043*	12 (27,9)	0,238
rs6801273 T/T	86 (36,6)	26 (38,2)		18 (41,9)	
rs6801273 T/C	123 (52,3)	31 (45,6)	0,331	22 (51,1)	0,608
rs6801273 C/C	26 (11,1)	11 (16,2)		3 (7,0)	
rs6801273 T/C+C/C	149 (63,4)	42 (61,8)	0,780	25 (58,1)	0,474
rs6798347 G/G	152 (64,7)	41 (59,4)		26 (60,5)	
rs6798347 G/A	72 (30,6)	27 (39,1)	0,175	15 (34,9)	0,828
rs6798347 A/A	11 (4,7)	1 (1,5)		2 (4,7)	
rs6798347 G/A+A/A	83 (35,3)	28 (40,6)	0,359	17 (39,5)	0,561

AMI – Acute myocardial infarction. 1 – Chi-square analysis between Control and AMI>45. 2 – Chi-square analysis between Control and AMI≤45. * $p \leq 0,05$.

For GP Ib platelet alpha subunit polymorphisms, no statistically, significant difference between Control and AMI groups was also found. For rs2243093 polymorphism we found a *p* value of 0,104 when AMI≤45 was compared with Control group. It is important to note that for this polymorphism only one homozygote (C/C) was found and corresponded to one control individual. For rs6065 polymorphism, as in the previous case, although the prevalence of heterozygotes C/T was higher in AMI≤45 (26,2%) compared with Control group (18,5%), the chi-square test *p* value was not statistically significant (*p*=0,199). No homozygotes (T/T) were found for this polymorphism in the whole studied population. When we divided our Control group in two subpopulations considering age (Control≤45 years and Control>45 years) and include only males, rs6065 is the only polymorphism that showed statistical differences in these conditions. In this case, we found a chi-square test *p* value lower than 0,001 when compared Control≤45 only males (n=44) with AMI≤45 only males (n=38). Distribution of genotypes were 41 C/C and 3 C/T for Control≤45 only males and 27 C/C and 11 C/T for AMI≤45 only males. When we calculate odds ratio for C/T *vs*. C/C genotypes in AMI≤45 only males, a *p*=0,016 was found (OR 5,568; 95% CI 1,421-21,822). No adjusted odds ratio was calculated due to the low numbers found in these conditions (table IV).

With respect to the other GP Ib platelet alpha subunit polymorphism studied, the VNTR, no differences were found in chi-square tests (table III). In our study population, no A allele for VNTR was found.

For the integrin subunit alpha 2 polymorphism, rs938043469, a significant difference was found in chi-square test *p* value when AMI>45 was compared with Control group (*p*=0,011). This significant difference was mainly due to an increase in TT homozygotes (19,2% in AMI>45 vs. 9,9% in Control) in detriment of CC homozygotes (28,2% in AMI>45 vs. 38,7% in Control) (table III). Accordingly, the odds ratios for AMI>45 was statistically higher in TT *vs*. CC genotypes (OR 2,662, CI 1,181-6,001, *p*=0,027) (table IV). After adjustment, OR maintain its statistical significance, which means that patients with a TT genotype have an increased risk of having an AIM>45years independently of the other variables (OR 2,852, 95% CI 1,092-7,451, *p*=0,032)

(table IV). No differences were found concerning the comparison between Control group and AMI≤45 group for this polymorphism. When both patient groups were compared by means of a binary logistic regression analysis, patients with TT genotype were more likely to have a myocardial infarction after 45 years (OR=4,528, 95% CI=1,047-19,576, *p*=0,043).

For the three *P2RY12* studied polymorphisms, we found a significant difference in rs2046934 when AMI>45 was compared with Control group in associated genotypes, AG+GG (*p*=0,043). What account for this difference is an increase in heterozygotes in AMI>45 when compared with Control. However, when odds ratio test was performed to compare AA to AG+GG genotype, the *p* value wasn't statistically significant (OR 1,665, CI 0,933-2,970, *p*=0,090) (table IV).

Table IV. Odds ratio from polymorphisms that showed a significant chi-square test analysis.

Odds ratios					
	Genotypes	**Odds ratio (95% CI)**	***p* value**	**Adjusted odds ratio (95% CI)**	***p* value**[1]
rs938043469 (Control vs. AMI>45)	TT vs. CC	2,662 (1,181-6,001)	0,027*	2,852 (1,092-7,451)	0,032*
rs2046934 (Control vs. AMI>45)	AG+GG vs. AA	1,665 (0,933-2,970)	0,090	-	-
rs6065 (Control≤45 vs. AMI≤45 and only males)	CT vs. CC	5,568 (1,421-21,822)	0,016*	-	-

AMI – Acute myocardial infarction. CI – Confidence interval. * p ≤ 0,05. 1 – Adjusted for the non-genetic variables studied and the other studied polymorphisms.

Discussion

In this study, we evaluated the effect of several platelet polymorphisms in patients that suffered acute myocardial infarction (AMI). These patients were divided in two groups, one formed by patients that suffered AMI with 45 years or earlier and another established by patients with more than 45 at the time that they suffered the first episode of AMI.

According to our results, the only polymorphism significantly associated with an increased risk for AMI in our entire studied population is rs938043469 from integrin subunit alpha 2 (GPIa) of integrin $\alpha 2\beta 1$. We can also conclude that the presence of the T allele in homozygosity is essential for the increased risk of AMI. Still, rs938043469 does not seem to be a polymorphism that influence early onset of AMI, since our results for AMI≤45 group showed no correlation between the presence of this polymorphism and AMI (table III). The frequency of the T/T genotype in our Control population (9,9%) was within the range of other previous reports (Santoso et al. 1999, Atherosclerosis 2003, Lu et al. 2014). In our AMI>45 group we found a rise in T/T genotype (19,2%) when compared to Control group. Since Kunicki et al first described this polymorphism in 1997 (Kunicki et al. 1997) many studies tried to assess the relationship between the polymorphism and cardiovascular diseases, mainly in younger adults, with controversial results (Carlsson et al. 1999, Santoso et al. 1999). In 2007, a meta-analysis of published data regarding the role of rs938043469 in coronary artery disease (CAD) concluded for the lack of association between this polymorphism and CAD (Tsantes et al. 2007). Nevertheless, our results were very similar to what Jian-Xia Lu et al found in Chinese patients that has suffered ischemic stroke (Lu et al. 2014), although the ethnic differences and different cardiovascular diseases. Moreover, a recent meta-analysis study found that T allele or the TT genotype of rs938043469 polymorphism was associated with increased risk for ischemic stroke (Liu et al. 2017).

Despite being a silent polymorphism, rs938043469 is believed to influence the density of integrin $\alpha 2\beta 1$ (GP Ia-IIa) in platelet membrane, being allele T responsible for the highest expression of the glycoprotein (Kritzik et

al. 1998, Corral et al. 1999). This variation in the receptor density may account for a higher platelet responsiveness to collagen in T/T genotype, enhancing the thrombotic potential of platelets in pathological states. However, it should be remembered that this receptor, although its importance for platelet adhesion to sub endothelial collagen mainly in static conditions, it is not physiologically as important as GP Ib-V-IX for the initiation of adhesion to sub endothelial tissue in conditions of high shear stress as found in arterial circulation (Brass et al. 2007). This can explain the difficulty of highlight a statistical pivotal role for this polymorphism in some cardiovascular diseases. The conflicting results found may also reflect different study designs (different patients and diseases (Carlsson et al. 1999, Santoso et al. 1999, Atherosclerosis 2003, Lu et al. 2014, Liu et al. 2017)) and the statistical difficulty of assert firmly that a single polymorphism in itself is crucial for such a complex and multifactorial disease as acute myocardial infarction.

In many studies, including the present one, some results fall in statistical limits that prevent us from decisively conclude for any expected association but, in our opinion, these results should not be despised and must require a carefully analysis to try to find their biological meaning. Bearing this in mind, we should highlight rs2243093, rs6065, VNTR and rs2046934 polymorphism results in our AMI groups.

The rs2243093 polymorphism analysis reveal an increase in T/C genotype in AMI≤45 group when compared with Control and the chi-square test was very close to significance (p=0,104). A very similar result was found in two studies where the authors associated rs2243093 polymorphism with an increased risk of ischemic stroke (Baker et al. 2001, Esen et al. 2012). In these studies, increases of T/C genotype were reported (Baker et al. 2001, Esen et al. 2012)). Another study associating rs2243093 polymorphism and risk of coronary heart disease published in 2015 by Zhang J. et al found a high frequency of C/C genotype and concluded that the C/C genotype is a biomarker of genetic susceptibility (Zhang et al. 2015). Interestingly, we only found one individual with C/C genotype in Control group. This means a frequency of 0,4% of the total population, what is within the range of another populations (Afshar-Kharghan et al. 1999). This polymorphism lies in 5'UTR of

GP1BA gene, where a T or a C may be present in position -5 from ATG start codon. Allele C was associated with increased expression of the receptor on the cell membrane (Afshar-Kharghan et al. 1999), what might explain the link between this polymorphism and a more pronounced potential of arterial thrombus formation in some vascular pathologies due to a higher platelet adhesiveness. Nevertheless, our results did not show a clear correlation between Kozak polymorphism and acute myocardial infarction, in any AMI group studied.

rs6065 polymorphism is based on the presence of threonine or methionine at position 161 of GP Ib platelet alpha subunit due to a C1018T nucleotide change (Kuijpers et al. 1992). rs6065 is of clinical importance because it is implicated in neonatal alloimmune thrombocytopenic purpura, posttransfusion purpura, and refractoriness to HLA-matched platelet transfusion (Kiefel et al. 2001). The presence of methionine was associated with an increased risk for myocardial infarction and CAD in several studies (Mikkelsson et al. 2001, Zhang et al. 2015). However, in an effort to study the functional effects of this polymorphism, Ulrichts H. et al in 2003 described a stronger interaction between GP Ib platelet alpha subunit and von Willebrand factor in glycoproteins that carries threonine (C allele) than methionine (T allele) (Ulrichts et al. 2003). This finding does not agree with the previous described results. Nevertheless, as the authors highlight in their paper, vWF binding to GP Ib platelet alpha subunit was investigated in a stationery state and not in shear stress conditions usually seen in a vascular stenosis (Ulrichts et al. 2003). We found a higher percentage of heterozygotes C/T when compared with Control group in our AMI≤45 group. In AMI>45 group no differences were found when compared with Control group. These results, although not statistically significant, are in accordance with the findings of Mikkelsson J. et al where they described a correlation between the presence of Met161 and sudden cardiac death in men younger than 55 years (Mikkelsson et al. 2001). Interestingly, when we ran the statistics and include only men in our Control≤45 and AMI≤45 groups, we found a significant difference ($p<0,001$) in genotypes distribution, with a higher percentage of heterozygotes in AMI≤45 compared with Control≤45. This result, despite the low *n*

implicated, mainly in Control≤45, highlight the possible contribution of rs6065 polymorphism to acute myocardial infarction of early onset.

For VNTR polymorphism in GP Ib platelet alpha subunit we did not find any A allele (four repeats) in our study groups and found one rare E allele, which has only been described in Caucasians (Ozelo et al. 2004). Allele A has been described mainly in Japanese and North America Indian populations (Aramaki and Reiner 1999). The most relevant results that we observed relating this polymorphism is the higher percentage of VNTR B/C genotype in AMI≤45 when compared with Control and AMI>45 groups. However, the chi-square test *p* value was not significant. This may be due to the large number of classes (six genotypes) and our sample size, mainly in AMI≤45 group. The literature often gauges a protective role to C/C genotype (Afshar-Kharghan et al. 2004) and we draw attention for the fact that C/C genotype was higher in Control group than in AMI>45 group and in AMI≤45 group. Studies targeted to assess the physiological response of each one of the different sized GP Ib platelet alpha subunit are mandatory, and in one attempt to help clarify this issue we conducted a study where platelets with different VNTR genotypes were subjected to PFA-100® tests using Collagen/ADP and Collagen/Epinephrine cartridges and occlusion time was measured. Our preliminary results showed that platelets with C/C genotype take longer to occlude the cartridge than any platelets that bear a B allele (B/C or B/D), possibly confirming the hypothetical protective status previously reported (data not shown).

For rs2046934 polymorphism we found a statistical significant chi-square test *p* value. This difference was found when AG and GG genotypes were associated and, once more as in C807T polymorphism, the difference was found in AMI>45 group. When associated, the difference is due to an increase in heterozygotes in AMI>45 relative to Control. rs2046934 is an intronic polymorphism that do not alter any amino acid and it is in linkage disequilibrium with other polymorphisms which also do not change the protein structure of the receptor. All these polymorphisms form the so-called H2 haplotype of the *P2RY12* gene (Fontana et al. 2003). In aggregometry tests, the H2 haplotype was associated with higher maximal aggregation in response to ADP when compared with H1 haplotype (Fontana et al. 2003). This result

suggested a hypothetical relationship between H2 haplotype and arterial thrombosis. However, Amisten S. et al conducted a study that found no association between H2 haplotype and AMI (Amisten et al. 2008). In our present study, although the differences found in the chi-square test, when the *p* value of an odds ratio test was calculated for A/G+G/G vs. A/A genotypes in AMI>45 group, no statistical significance was found. In a recent study meant to evaluate the effects of platelet polymorphisms on antiplatelet drug responsiveness and clinical outcomes in patients with acute minor ischemic stroke, that include the *P2RY12* gene, the authors fail to address a potential pathophysiological role to any of the *P2RY12* polymorphisms that we also studied (Yi et al. 2017). Additional studies should be conducted to elucidate the pathophysiological role, if any, of rs2046934 polymorphism. For the other *P2RY12* polymorphisms studied no statistical differences were found.

The rs5918 polymorphism showed no correlation or any trend with acute myocardial infarction in any AMI group. Despite many studies suggested an association between Pl^{A2} (C allele) and arterial events (Weiss et al. 1996, Grove et al. 2004), we didn't found any statistical correlation between this polymorphism and AMI. This lack of statistical difference was also reported in other several studies (Herrmann et al. 1997, Aleksic et al. 2000) and contradiction relating the relationship between this polymorphism and cardiovascular pathology remains.

The results of the present work, despite of its inherent limitations, namely a limited number of patients in our AMI groups and the heterogeneity between control and AMI groups of patients, provide important hints on the possible role of platelet receptor polymorphisms in thrombotic disease. The most important conclusion of our work is that genotype T/T from rs938043469 polymorphism from platelet integrin subunit alpha 2 (GPIa) is significantly associated with the presence of AMI in patients older than 45 years of age. Further studies with larger and more homogenous cohorts may enhance our findings and will help to clarify the role of rs6065 and rs2046934 polymorphisms and their association with AMI. It is important to point out that both groups of AMI patients differ also in non-genetic variables, so, further studies are mandatory to understand the molecular pathways associated to the

polymorphisms studied. Also, the association between these polymorphisms and the clinical outcome of AMI groups should be studied to establish a better stratification of AMI patients in function of their genetic profile.

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Conflict of interest

The authors report no declarations of interest.

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Artigo 2

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NOS3 gene polymorphism rs2070744 is a risk factor for acute myocardial infarction in patients with less than 45 years.

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Abstract

Introduction: We examined the potential role of *NOS3* gene polymorphisms rs2070744 and rs1799983 as risk factors for the development of acute myocardial infarction (AMI). Material and methods: The studied population was divided in 3 groups: Control (n=235), AMI≤45 years (n=44) and AMI>45 years (n=78). Polymorphisms were detected by PCR (polymerase chain reaction) and RFLP (restriction fragment length polymorphisms) analysis. Results: Regarding non-genetic risk factors, the Control group differed statistically from AMI group≤45 years (p <0,05) in terms of smoking habits, hypertension, diabetes mellitus and obesity, and differed statistically from the AMI group>45 years (p <0.05) in the variables hypertension, diabetes mellitus, obesity, family history of thrombosis and high cholesterol. For rs2070744 polymorphism a statistical difference was found when AMI≤45 was compared with Control group, with a higher risk of AMI in TC genotype (odds ratio 9,313; Confidence interval 95% from 1,764 to 49,170; p=0,009). The other polymorphism studied did not show any statistical significant correlation. Conclusion: There is a statistically association between TC genotype from rs2070744 polymorphism and increased risk for AMI≤45.

Keywords

Acute myocardial infarction; *NOS3* gene; rs2070744; rs1799983.

Introduction

Endothelial nitric oxide synthase (eNOS) is one of the four existing NOS enzymes capable of produce nitric oxide (NO), the others being inducible NOS (iNOS), neural NOS (nNOS) and mitochondrial NOS (mtNOS) (Rex and Freedman 2007). When nitric oxide (NO), a gas with multiple physiological functions, is released by eNOS in cardiovascular system, it acts as a platelet inhibitor and a vasodilator, thus helping to regulate primary hemostasis activation and vascular tone (Rex and Freedman 2007). Until now, eNOS expression in a constitutive manner was described in many different type of cells, including endothelial cells (Nathan 1992), cardiomyocytes (Michel and Feron 1997), bronchiolar epithelial cells (German et al. 2000), megakaryocytes and, consequently, platelets (Sase and Michel 1995). Several polymorphisms in eNOS gene (*NOS3*) has been described, either in promoter, intronic or exonic regions (Rex and Freedman 2007). Single nucleotide polymorphism (SNP) rs2070744 (formerly known as -786T>C) in the promoter region, associated with reduced eNOS expression (Nakayama et al. 1999), and SNP rs1799983 (formerly known as 894G>T) in exon 7 that leads to an amino acid substitution at residue 298 (Glu>Asp) (Hingorani et al. 1999), were both associated in several studies with disease states, namely, myocardial infarction (Hingorani et al. 1999), coronary artery disease (Rossi et al. 2003), carotid stenosis (Ghilardi et al. 2002), hypertension (Miyamoto et al. 1998, Hyndman et al. 2002) and coronary spasm (Chang et al. 2003). However, lack of association between these polymorphisms and some of the referred disease states was also reported (Benjafield and Morris 2000, Sigusch et al. 2000, Granath et al. 2001). In the present work, we studied the potential role of rs2070744 (-786T>C) and rs1799983 (894G>T) *NOS3* gene polymorphisms as risk factors for the development of acute myocardial infarction. For that, two groups of patients who suffered acute myocardial infarction (AMI) were considered according to their ages: one with less than 45 years and another with more

than 45 years.

Material and methods

Population

The studied population included only Portuguese Caucasians. Donors were divided in 3 groups designated as Control, AMI≤45 years and AMI>45 years. Control group consisted of 235 donors (57 males and 178 females) with ages ranging from 15 to 69 years old (mean 27 years). At the time of the sample collection donors had no clinical signs of any hemorrhagic or thrombotic disease. AMI≤45 group consisted of 44 donors (40 males and 4 females) that suffered an acute myocardial infarction at, or before, 45 years, with ages ranging from 27 to 45 years old (mean 42 years). AMI>45 group consisted of 78 donors (67 males and 11 females) that suffered an acute myocardial infarction after 45 years, with ages ranging from 51 to 97 years old (mean 67 years). Clinical data regarding smoking status, blood pressure, presence of diabetes, body mass index, family history of cardiovascular disorders and total cholesterol levels were collected for the 3 groups. Previously, the genotype for platelet receptor polymorphisms was assessed in the three groups (data not shown). All donors gave written informed consent to the protocol, which was approved by the local ethics committee.

Genotype analysis

Genomic DNA was extracted from peripheral blood cells collected by venipuncture in EDTA tubes using a PureLink™ Genomic DNA Mini Kit (Invitrogen). A region containing each polymorphism was amplified by PCR using 1μg of DNA and 1μM of specific primers. Primers for rs2070744 (-786T>C) were F5`GTGTACCCCACCTGCATTCT3` and R5`CCCAGCAAGGATGTAGTGAC3` and for rs1799983 (894G>T) were F5`AGCCTCGGTGAGATAAAGGA3` and R5`CCAATTTCCAGCAGCATGT3`. Amplicons were then digested with specific restriction enzymes, BsuRI (HaeIII) for rs2070744 (-786T>C) and MboI for rs1799983 (894G>T), and the digested fragments were visualized in a 2% ethidium bromide agarose gel.

Statistical analysis

Genotypic and allelic frequencies for the Control group were calculated and Hardy-Weinberg equilibrium was tested for both polymorphisms by means of a chi-square test using observed vs. expected genotypic frequencies. Genotypic frequencies from AMI>45 and AMI≤45 groups were compared with the observed genotypic frequencies obtained from Control group using a chi-square test. For those chi-square tests that presented a *p* value ≤0,05, odds ratios (OR) with 95% confidence intervals (CI) were calculated as estimates of the development of acute myocardial infarction as a function of the studied polymorphism. Binary logistic regression analysis was performed to determine the association between AMI and the studied variables through calculation of OR and their 95% CI. Adjusted OR was estimated for the physiological profile, using Multinomial logistic regression (adjusted maximum-likelihood estimation). Enter method was selected to enter independent variables into the analysis. IBM SPSS Statistics for Windows, Version 24.0 software was used for all statistical calculations.

Results

Table I shows the clinical data for the three studied groups. As can be seen in table I, we found differences between AMI≤45 and Control group in the following variables: smoking status (OR=5,651; CI 95% 2,2472-14,2041; *p*<0,001), hypertension (OR=16,755; CI 95% 4,521-62,500; *p*<0,001), diabetes mellitus (OR=60,948; CI 95% 3,644-1019,274; *p*=0,004) and obesity (OR= 5,6515; CI 95% 2,2472-14,2041; *p*<0,001) and between Control group and AMI>45: hypertension (OR=58,823; CI 95% 17,544-200,000; *p*<0,001), diabetes mellitus (OR=80,119; CI 95% 4,812-1334,049; *p*=0,002), obesity (OR=3,472; CI 95% 1,013-11,905; *p*=0,048), family history of thrombosis (OR=8,707; CI 95% 2,657-28,532; *p*<0,001) and elevated cholesterol (OR=3,891; CI 95% 1,292-11,765; *p*<0,016). Also, there is a significant heterogeneity in gender distribution among the studied groups (*p*<0,001 for both AMI groups), with a predominant number of males in both groups of patients.

Table I. Clinical characteristics of studied subjects.

	Control	AMI≤45	AMI>45
Number of subjects	235	44	78
Gender (male), n (%)	57 (24,3)	40 (90,9)*	67 (85,9)*
Active smokers, n (%)	62 (26,3)	30 (68,2)*	27(34,6)
Hypertension, n (%)	9 (3,8)	16 (36,4)*	54 (69,2)*
Diabetes mellitus, n (%)	0 (0,0)	10 (22,7)*	22 (28,2)*
Obesity (BMI above 30 kg/m^2), n (%)	8 (3,4)	11 (25,0)*	19 (24,4)*
Family history of thrombosis, n (%)	107 (45,5)	25 (56,8)	6 (7,7)*
Total cholesterol above 200 mg/dL, n (%)	59 (25,1)	11 (25,0)	43 (55,1)*

AMI – acute myocardial infarction. BMI – body mass index. Chi-square test was used to compare variables among control and AMI groups. * p value <0,05 when compared to Control group.

Genotypes were in Hardy-Weinberg equilibrium for both polymorphisms in the Control group. Table II shows the genotypic frequencies for the polymorphisms in the 3 groups studied and the chi-square *p* value obtained when each AMI group was compared with Control group for each polymorphism. As can be seen in table II, the chi-square *p* values for both polymorphisms were statistically significant when AMI≤45 group was compared with Control group, which means that genotypic distribution of AMI≤45 group differ from genotypic distribution of Control group (*p*=0,006 for rs2070744 and *p*=0,011 for rs1799983). The difference found in rs2070744 was mainly related with a decrease in TT genotype (28,1% in Control group vs. 7,1% in AMI≤45) with a concomitant increase of TC genotype (52,6% in Control group vs. 73,8% in AMI≤45), with no change in CC genotype frequency. In rs1799983, the difference found was due to an increase of TT genotype (13,9% in Control group vs. 29,5% in AMI≤45) at the expense of both GG and GT genotypes. No statistical differences were found when the same comparisons were made between Control and AMI>45 groups. In table II, also, we can see that odds ratio in rs2070744 polymorphism, in AMI≤45 group, was statistically significant in TC genotype vs. TT genotype (OR 6,203, 95% CI

1,776-21,670, *p*=0,004). In rs1799983 polymorphism, again in AMI≤45 group, odds ratio was statistically significant in TT genotype vs. GG genotype (OR 2,492, CI 1,071-5,798, *p*=0,040). After adjustment, only rs2070744 polymorphism OR maintain its statistical significance, which means that patients with a TC genotype have an increased risk of having an acute myocardial infarction before 45 years, independently of the other studied variables (OR 9,313, 95% CI 1,764-49,170, *p*=0,009) (table II).

Table II. Genotypic frequencies and odds ratios for rs2070744 (-786T>C) and rs1799983 (894G>T) polymorphisms.

Polymorphism genotypes		**Control, n (%)**	**AMI>45, n (%)**	***p* value**[1]	**AMI≤45, n (%)**	***p* value**[2]
rs2070744	TT	64 (28,1)	23 (29,9)	0,971	3 (7,1)	0,006*
	TC	120 (52,6)	37 (48,0)		31 (73,8)	
	CC	44 (19,3)	17 (22,1)		8 (19,1)	
rs1799983	GG	92 (39,8)	31 (39,7)	0,552	15 (34,1)	0,011*
	GT	37 (46,3)	33 (42,3)		16 (36,4)	
	TT	32 (13,9)	14 (18,0)		13 (29,5)	

Odds ratios					
	Genotypes	**Odds ratio (95% CI)**	***p* value**	**Adjusted odds ratio (95% CI)**	**Adjusted *p* value**[3]
rs2070744 AMI≤45	TC vs. TT	6,203 (1,776-21,670)	0,004*	9,313 (1,764-49,170)	0,009*
	CC vs. TT	3,595 (0,850-15,212)	0,082	3,488 (0,512-23,775)	0,202
rs1799983 AMI≤45	GT vs. GG	0,917 (0,430-1,956)	0,849	0,986 (0,367-2,646)	0,977
	TT vs. GG	2,492 (1,071-5,798)	0,040*	3,504 (0,897-13,682)	0,071

AMI – Acute myocardial infarction. CI – Confidence interval. 1 – Chi-square analysis between Control and AMI>45. 2 – Chi-square analysis between Control and AMI≤45. * p ≤ 0,05. 3 – Adjusted for the non-genetic variables studied.

Discussion

In this study, we evaluated the effect of two eNOS polymorphisms (rs1799983 and rs2070744) in patients that suffered acute myocardial infarction (AMI). These patients were divided in two groups, one formed by patients that suffered AMI with 45 years or earlier and another established by patients with more than 45 at the time that they suffered the first episode of

AMI. According to our results, rs2070744 polymorphism is significantly associated with an increased risk for AMI before 45 years. Our results also show that TC genotype is associated with AMI before 45 years, which means that the presence of one C allele is sufficient to increase the risk for early events of AMI. Noteworthy, we found a similar allelic frequency of the C allele in our Control and AMI>45 groups (45,6% and 46,1%, respectively), while in AMI≤45 that allelic frequency rose to 56,9%. The association of this polymorphism with cardiovascular pathologies of early onset in literature is unclear, and different results have been reported. In 2001, Granath B. et al fail to address a relation between rs2070744 polymorphism and premature coronary artery disease (CAD) in an Australian Caucasian population (Granath et al. 2001). In contrast with the previous report, Alvarez R. et al found an association between this polymorphism and early CAD (Alvarez et al. 2001). rs2070744 is a polymorphism where the promoter activity of *NOS3* gene is severely reduced (Nakayama et al. 1999), leading to a reduced NO production, either by the endothelium or by platelets. It has been hypothesized that lack of NO production by the endothelium leads to artery vasospasm (Nakayama et al. 1999) and fails to limit the oxidation of atherogenic low-density lipoprotein (LDL) (Rubbo and O'Donnell 2005), both of which can contribute to coronary artery disease. To note that in our AMI≤45 group, 68,2% were active smokers at the time they suffered their first episode of AMI, being smoking one of the major risk factors for coronary spasm (Yoo and Kim 2009). We separate the impact of smoking from the effect of rs2070744 polymorphism in AMI≤45 (adjusted OR, p=0,009). In this sense, Wang J. et al pointed out that the addictive effect of smoking and the presence of rs2070744 may greatly enhance the chances of suffer an AMI before 45 years of age (Wang et al. 2002). In addition to the role of NO to prevent vasospasm, to limit the oxidation of LDL and to inhibit platelet activation in normal blood vessels (de Graaf et al. 1992), it has been addressed its role in modulating platelet function, either at rest (Zhou et al. 1995) or during aggregation (Freedman et al. 1997, Randriamboavonjy and Fleming 2005). In a recent study, Radziwon-Balicka A. et al described the existence of two platelet subpopulations concerning their ability to produce NO, one that lacks eNOS, and thus cannot synthesize NO,

and other that possesses eNOS and are capable of produce and release NO (Radziwon-Balicka et al. 2017). This latter subpopulation seems to have an important role to limit the growing and the size of a platelet aggregate. All this four NO actions (prevent vasospasm, limit the oxidation of LDL, inhibit circulating platelets and modulate the formation of a platelet aggregate), may contribute to the explanation why this polymorphism, where the NO levels are reduced, can lead to cardiovascular diseases such as AMI before 45 years of age and other cardiovascular events, previously linked to low levels of NO, like delayed cerebral ischemia following aneurysmal subarachnoid hemorrhage (Hendrix et al. 2017).

The rs1799983 polymorphism analysis revealed an increase in TT genotype in our AMI≤45 group compared with Control. Also, the allelic frequency of T allele in AMI≤45 group was higher than in Control and AMI>45 groups (47,7% vs. 37,0% and 39,1%, respectively), which highlights the possible importance of this polymorphism in early events of AMI. Although the chi-square analysis between Control and AMI≤45 was statistically significant, when adjusted OR was performed, the *p* value found was very close to significance but above its limit (p=0,071). Given the inherent limitations of this study, namely a limited number of patients in our AMI groups, a carefully analysis of the obtained results should be considered. The rs1799983 polymorphism consists in an amino acid substitution in position 298, where glutamate is replaced by aspartate, due to a nucleotide substitution in position 894 G>T. The T allele was linked with endothelial disfunction, even in young men without any cardiovascular disease (Imamura et al. 2004, Godfrey et al. 2007). These results were partially corroborated by studies where endothelial-dependent vasodilation was assessed in patients with hypertension condition. In this case, the effect of rs1799983 polymorphism alone wasn't statistically significative, but enhanced the effect of the other polymorphism studied, coincidentally, rs2070744 polymorphism, which showed a significant independent effect in that study (Rossi et al. 2003). T allele of this polymorphism was also associated with worse prognosis of a group of patients who suffered ST-elevation myocardial infarction and had low levels of soluble CD40 ligand (sCD40L), high concentrations of N-terminal pro-brain natriuretic

peptide (NT-pro-BNP) and higher expression of specific microRNAs, namely miR-19B, miR145 and miR222 (Napoleao et al. 2016). In this last study, the polymorphism seems to contribute to impair cardiac and endothelial recovery. Finally, and considering the inherent limitations of our study, that consists, chiefly, in a limited number of patients in our AMI groups and the heterogeneity between control and AMI groups, we can conclude that C allele of rs2070744 polymorphism seems to be associated with an increased risk for acute myocardial infarction in patients younger than 45 years of age. Regarding rs1799983 polymorphism our results failed to address any association with risk for AMI. Further studies are mandatory on the molecular mechanisms, but our results suggest that the assessment of eNOS polymorphism profile could be a useful tool for cardiovascular risk screening.

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Capítulo 4: Seleção positiva de aminoácidos na integrina plaquetária αIIbβ3 em diferentes espécies de mamíferos

Artigo 1

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Maximum likelihood approach suggests positive selection in platelet integrin αIIbβ3 in mammalian species.

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Abstract

Platelet integrin αIIbβ3 is crucial for platelet aggregation. Although structural and functional characteristics of this protein have been extensively studied, the evolutionary pattern studies of this protein complex in mammals are scarce. Here, we addressed this question using maximum likelihood approaches to identify codons that are evolving under positive selection. Likelihood of positive selection was estimated using CODEML implemented in PAML software applied to integrin αIIbβ3 derived from nucleotide sequences of ten different mammalian species. Four codons in mature αIIb-subunit (corresponding to residues 150, 184, 193 and 370) and three codons in mature β3-subunit (corresponding to residues 129, 440 and 444) showed signs of positive selection with posterior probabilities over 95%. The different amino acids observed for each of the positively selected residues detected showed different physicochemical properties. These results open new research avenues to understand the physiological importance of specific residues and should allow for a better understanding of the function and the different interactions of each residue within the mature protein.

Keywords

Positive selection, platelet integrin αIIbβ3.

Introduction

Platelet integrin αIIbβ3, commonly known as platelet GPIIb-IIIa, is one of the most highly expressed platelet cell membrane receptors (17% of all cell membrane proteins) and is responsible for platelet aggregation (Wagner et al. 1996). It is also expressed in other platelet membrane compartments, namely platelet α granules and open-canalicular membranes. This integrin reservoir fuses with the cell membrane after platelet activation (Buensuceso et al. 2003). In resting platelets αIIbβ3 has low affinity for its ligands. After initial platelet activation it undergoes a conformational change regulated by a mechanism of inside-out signalling, promoting an increase on its affinity for ligands such as divalent fibrinogen or multivalent von Willebrand factor (vWF). In this activated state, specific ligands can now bind to αIIbβ3 integrins that act like adhesive points for platelet-platelet interactions, leading to a second wave of platelet signalling and, most importantly, to platelet aggregation, suspending blood loss where vascular injury had happened (Savage et al. 2001).

In humans, many genetic polymorphisms have been described within the αIIb-subunit (*ITGA2B*) and the β3-subunit (*ITGB3*) genes, being polymorphism Pl^A (rs5918), composed of two allelic forms, (A1) and (A2), one with clinical significance because it can cause neonatal alloimmune thrombocytopenia, mainly in Caucasians (Williamson et al. 1998). Also, mutations within any of these genes lead to a bleeding disease known as Glanzmann thrombasthenia (French 1998). Each protein subunit contains an extracellular domain, a transmembrane region and a short cytoplasmic tail (Xiao et al. 2004). The extracellular domain of αIIb-subunit is composed of a β-propeller with seven blades that contain four divalent ion-binding sites (Xiong et al. 2001), a thigh domain and two calf domains (calf-1 and calf-2). The thigh and calf domains are arranged into a β-sheet (Plow et al. 2007). The extracellular domain of β3-subunit comprise a I-domain that contains three metal sites for binding ligands (Xiong et al. 2002), a Hybrid domain that

resembles the I-set immunoglobulin domains (Chothia and Jones 1997), a PSI (plexin-semaphorin-integrin) domain that connects the Hybrid domain to the first (EGF-1) of four EGF (Epidermal Growth Factor) domains and a □-tail domain near to the membrane (Xiong et al. 2001). The extracellular domains of the α and β subunits contact with each other mainly between the β-propeller and I-domain (Xiong et al. 2001). The αIIb cytoplasmic tail consists of 20 residues that are highly conserved among integrin α-subunits (Vinogradova et al. 2000). The β3 tail consists of 47 residues that form several α-helices (Vinogradova et al. 2004). The cytoplasmic tails of αIIb and β3 subunits interact with each other through their membrane proximal helices (Vinogradova et al. 2002).

Despite the crystal structure of αIIb and β3 subunits have been described (Chothia and Jones 1997, Vinogradova et al. 2000, Xiong et al. 2001, Vinogradova et al. 2002, Xiong et al. 2002, Vinogradova et al. 2004, Xiao et al. 2004, Plow et al. 2007), full understanding of their entire function is far from resolved. For instance, beyond its capacity to bind to vitronectin, the αVβ3 integrin was also associated with enhanced angiogenesis after HIV-1 infection of T-cells, a process mediated by binding of Tat protein of human immunodeficiency virus type-1 to endothelial αVβ3 integrin (Barillari et al. 1999). In this way, knowledge of the function and the different interactions of each residue within the mature protein may help to elucidate major αIIb and β3 physiologic properties. Here, we study the evolution of *ITGA2B* and *ITGB3* in mammals using maximum likelihood approaches to identify codons under positive selection.

Methods

The coding sequence for ααIIb-subunit is located on the long arm (q) of chromosome 17 (17q21.32), spanning 17324 bp. This gene (*ITGA2B*) contains 30 exons that will yield a single polypeptide precursor with 1039 residues which will undergo proteolytic cleavage into a heavy (871 residues) and a light chain (137 residues) linked by a disulphide bond. This mature protein is composed of 1008 residues (Heidenreich et al. 1990, Mikkelsson et al. 2001). The coding sequence for β3-subunit (*ITGB3* gene) is adjacent to the *ITGA2B*

gene on chromosome 17, spanning 58870 bp. It is composed by 15 exons that will yield a protein precursor with 788 residues. After an initial cleavage of 26 residues, the mature protein with 762 residues joins αIIb-subunit to form integrin αIIbβ3 which is incorporated into the membranes in its inactivated state (Zimrin et al. 1990, Ozelo et al. 2004). The complete sequences of mature integrins αIIb and β3 from 10 mammalian species were retrieved from GenBank (see figures 1 and 2) and were aligned using BioEdit software version 7.2.0 (Hall 1999).

Under neutrality, the expected ratio (ω) of non-synonymous (d_N) to synonymous (d_S) substitutions in a gene is one and significant deviations from this value can be interpreted as evidence of either positive (ω > 1) or purifying selection (ω < 1). However, this approach is highly conservative. Here, detection of positive selection was performed using the CODEML implemented in PAML (version 4) (Yang 2007). This maximum likelihood (ML) method, instead of averaging ω across the gene, uses codon-based models that allow heterogeneity in d_N/d_S among codons in a phylogenetic context, allowing detection of recurrent positive selection occurring only in a small portion of a gene. In CODEML, the M7 model only allows codons to evolve neutrally or under purifying selection while M8 adds a class of sites under positive selection. The two previous nested models were compared using the likelihood ratio test (LRT) with 2 degrees of freedom (Yang et al. 2000). Residues under selection for M8 were identified using Bayes Empirical Bayes approach (BEB) with posterior probability >95%. For each gene, a neighbor-joining tree was used as the working topology which was constructed using Mega7 with the options *p*-distance as the substitution model and complete deletion to gaps and missing data (Kumar et al. 2016).

Results

Evidence of positive selection in *ITGA2B* and *ITGB3* genes sequence was detected using a ML method implemented in the software package PAML (Yang 1997). We found that the comparison M7 with M8 is significant and models permitting positive selection are a better fit to the data. The LRT test showed

a value of 16.00 (p<0.001) and 14.02 (p<0.001) for αIIb and (β3), respectively. Regarding integrin αIIb we found four codons under positive selection (residues 150, 184, 193 and 370) with posterior probabilities over 95%. For each codon, six, five, seven and six different amino acid possibilities were observed, respectively (see figure 1 and table I). In integrin β3 we found three codons (residues 155, 466 and 470) under positive selection with posterior probabilities over 95%. Five different amino acids were observed for codons 155 and 466, and seven were identified for position 470 (see figure 2 and table II). These positions correspond to the human protein precursors before cleavage of the signal peptides as described in (Heidenreich et al. 1990) and (Zimrin et al. 1990). After the cleavage of the signal peptide the numeration becomes 119, 153, 162 and 339 for human αIIb mature protein and 129, 440 and 444 for human β3 mature protein. The numeration used from now on to describe their positions in the integrin αIIbβ3 will refer first to the mature protein without the signal peptide and in parenthesis with the signal peptide. From the seven detected codons, at least three show already described single-nucleotide polymorphisms (SNP) in humans, namely residues 153 (184) and 339 (370) from αIIb-subunit and residue 129 (155) in β3-subunit. In residue 153 (184) a nonsynonymous missense SNP exists, where the most common residue, an arginine (R), is changed by a histidine (H) or a leucine (L) and was classified as rs752291386. In residue 339 (370) a synonymous SNP was described and classified as rs41308596. In β3-subunit, a nonsynonymous missense SNP was described in residue 129 (155), where a tryptophan (W) is changed by an arginine (R).

Table I. Residues found to be under positive selection in αIIb-subunit among ten mammalian species and their respective basic physicochemical properties.

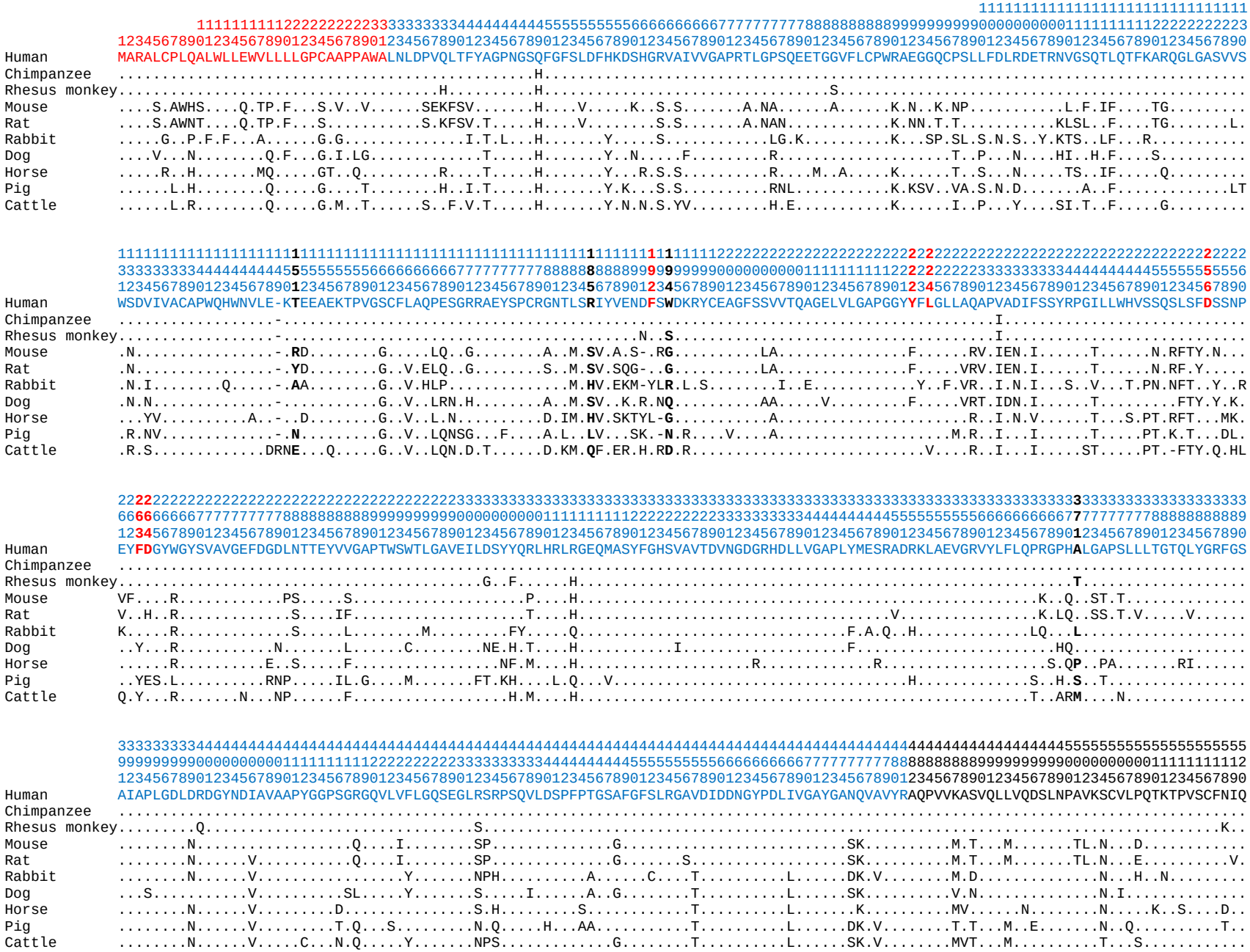
	Residue 119 (150)	**Residue 153 (184)**	**Residue 162 (193)**	**Residue 339 (370)**
Species				
Human (*Homo sapiens*)	T[1]	R[2]	W[7]	A[4]
Chimpanzee (*Pan troglodytes*)	T[1]	R[2]	W[7]	A[4]
Rhesus monkey (*Macaca mulata*)	T[1]	R[2]	S[1]	T[1]
Mouse (*Mus musculus*)	R[2]	S[1]	G[4]	A[4]
Rat (*Ratus norvegicus*)	Y[3]	S[1]	G[4]	A[4]
Rabbit (*Oryctolagus cuniculus*)	A[4]	H[6]	R[2]	L[4]
Dog (*Canis lupus familiaris*)	T[1]	S[1]	Q[1]	A[4]
Horse (*Equus caballus*)	T[1]	H[6]	G[4]	P[7]
Pig (*Sus scrofa*)	N[1]	L[4]	N[1]	S[1]
Cattle (*Bos taurus*)	E[5]	Q[1]	D[5]	M[4]

1 – Polar hydrophilic neutral; 2 – Basic polar hydrophilic positive; 3 – Polar neutral hydrophobic; 4 – Non-polar neutral hydrophobic; 5 – Acidic polar negative hydrophilic; 6 – Basic polar positive hydrophilic; 7 – Non-polar neutral hydrophobic. Residues numbers are without the signal peptide and in parenthesis with the signal peptide.

Table II. Residues found to be under positive selection in β3-subunit among ten mammalian species and their respective basic physicochemical properties.

	Residue 129 (155)	**Residue 440 (466)**	**Residue 444 (470)**
Species			
Human (*Homo sapiens*)	W[7]	Q[1]	N[1]
Chimpanzee (*Pan troglodytes*)	W[7]	Q[1]	N[1]
Rhesus monkey (*Macaca mulata*)	W[7]	Q[1]	N[1]
Mouse (*Mus musculus*)	S[1]	F[7]	S[1]
Rat (*Ratus norvegicus*)	S[1]	F[7]	L[4]
Rabbit (*Oryctolagus cuniculus*)	R[2]	H[6]	H[6]
Dog (*Canis lupus familiaris*)	S[1]	Q[1]	S[1]
Horse (*Equus caballus*)	G[4]	W[7]	Y[3]
Pig (*Sus scrofa*)	E[5]	Q[1]	D[5]
Cattle (*Bos taurus*)	R[2]	E[5]	F[7]

1 – Polar hydrophilic neutral; 2 – Basic polar hydrophilic positive; 3 – Polar neutral hydrophobic; 4 – Non-polar neutral hydrophobic; 5 – Acidic polar negative hydrophilic; 6 – Basic polar positive hydrophilic; 7 – Non-polar neutral hydrophobic. Residues numbers are without the signal peptide and in parenthesis with the signal peptide.


Human MARALCPLQALWLLEWVLLLLGPCAAPPAWALNLDPVQLTFYAGPNGSQFGFSLDFHKDSHGRVAIVVGAPRTLGPSQEETGGVFLCPWRAEGGQCPSLLFDLRDETRNVGSQTLQTFKARQGLGASVVS
Chimpanzee
Rhesus monkey
Mouse
Rat
Rabbit
Dog
Horse
Pig
Cattle
Human WSDVIVACAPWQHWNVLE-KTEEAEKTPVGSCFLAQPESGRRAEYSPCRGNTLSRIYVENDFSWDKRYCEAGFSSVVTQAGELVLGAPGGYYFLGLLAQAPVADIFSSYRPGILLWHVSSQSLSFDSSNP
Chimpanzee
Rhesus monkey
Mouse
Rat
Rabbit
Dog
Horse
Pig
Cattle
Human EYFDGYWGYSVAVGEFDGDLNTTEYVVGAPTWSWTLGAVEILDSYYQRLHRLRGEQMASYFGHSVAVTDVNGDGRHDLLVGAPLYMESRADRKLAEVGRVYLFLQPRGPHALGAPSLLLTGTQLYGRFGS
Chimpanzee
Rhesus monkey
Mouse
Rat
Rabbit
Dog
Horse
Pig
Cattle
Human AIAPLGDLDRDGYNDIAVAAPYGGPSGRGQVLVFLGQSEGLRSRPSQVLDSPFPTGSAFGFSLRGAVDIDDNGYPDLIVGAYGANQVAVYRAQPVVKASVQLLVQDSLNPAVKSCVLPQTKTPVSCFNIQ
Chimpanzee
Rhesus monkey
Mouse
Rat
Rabbit
Dog
Horse
Pig
Cattle

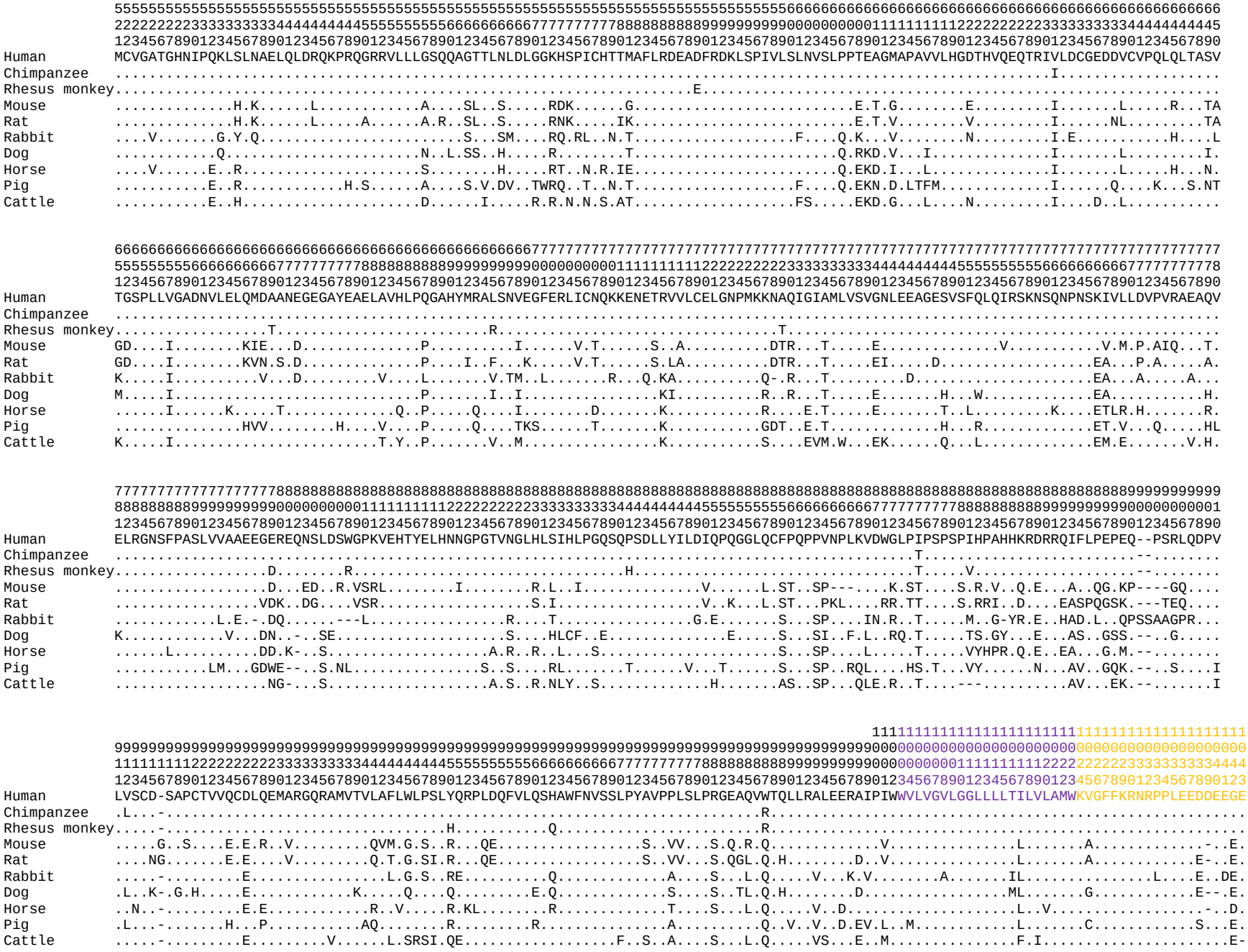
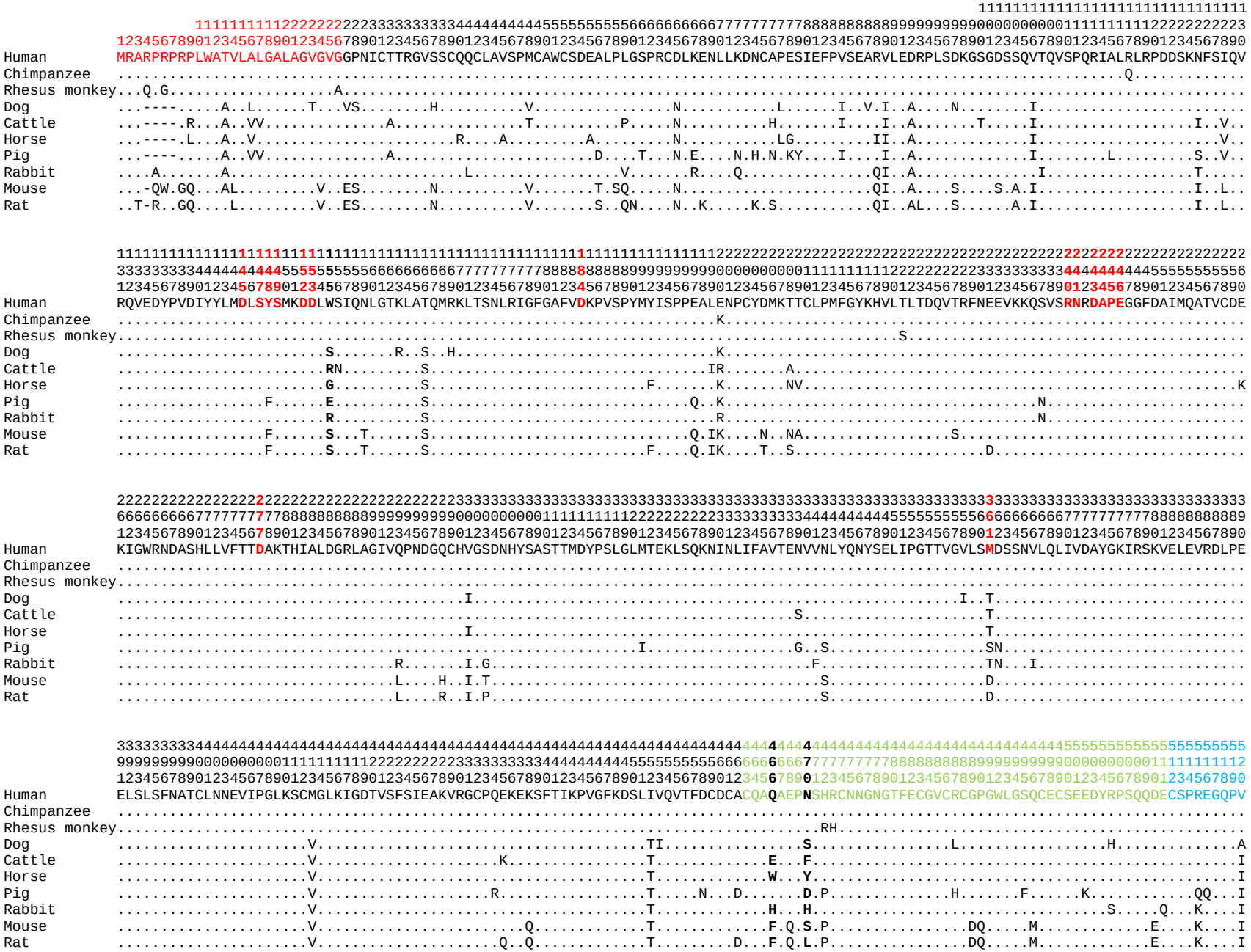

Human
Chimpanzee
Rhesus monkey
Mouse
Rat
Rabbit
Dog
Horse
Pig
Cattle

Figure 1. Alignment of integrin αIIb-subunit for 10 mammalian species. The entire human protein sequence is depicted. Signal peptide is in red. Amino acids under positive selection are in black bold. In red bold are depicted the residues that belong to the RGD-binding pocket [25]. In dark blue, β-propeller domain. In purple, the transmembrane domain. In orange, the cytoplasmic domain. Full stop points (.) represent the same amino acid as in human sequence. Dash marks (-) represent indels. Numbers above the sequences are read vertically from top to bottom and are only for help in alignment orientation, since there are four indels in human sequence. GenBank accession numbers: Human (Homo sapiens), NM_000419.4; Chimpanzee (Pan troglodytes), XM_016931653.1; Rhesus monkey (Macaca mulata), XM_001114526.3; Mouse (Mus musculus), NM_010575.2; Rat (Ratus norvegicus), XM_001063315.6; Rabbit (Oryctolagus cuniculus), NM_001082065.1; Dog (Canis lupus familiaris), NM_001003163.2; Horse (Equus caballus), NM_001081793.1; Pig (Sus scrofa), NM_213998.1; Cattle (Bos taurus), NM_001014929.1.


Human MRARPRPRPLWATVLALGALAGVGVGGPNICTTRGVSSCQQCLAVSPMCAWCSDEALPLGSPRCDLKENLLKDNCAPESIEFPVSEARVLEDRPLSDKGSGDSSQVTQVSPQRIALRLRPDDSKNFSIQV
Chimpanzee
Rhesus monkey
Dog
Cattle
Horse
Pig
Rabbit
Mouse
Rat
Human RQVEDYPVDIYYLMDLSYSMKDDLWSIQNLGTKLATQMRKLTSNLRIGFGAFVDKPVSPYMYISPPEALENPCYDMKTTCLPMFGYKHVLTLTDQVTRFNEEVKKQSVSRNRDAPEGGFDAIMQATVCDE
Chimpanzee
Rhesus monkey
Dog
Cattle
Horse
Pig
Rabbit
Mouse
Rat
Human KIGWRNDASHLLVFTTDAKTHIALDGRLAGIVQPNDGQCHVGSDNHYSASTTMDYPSLGLMTEKLSQKNINLIFAVTENVVNLYQNYSELIPGTTVGVLSMDSSNVLQLIVDAYGKIRSKVELEVRDLPE
Chimpanzee
Rhesus monkey
Dog
Cattle
Horse
Pig
Rabbit
Mouse
Rat
Human ELSLSFNATCLNNEVIPGLKSCMGLKIGDTVSFSIEAKVRGCPQEKEKSFTIKPVGFKDSLIVQVTFDCDCACQAQAEPNSHRCNNGNGTFECGVCRCGPGWLGSQCECSEEDYRPSQQDECSPREGQPV
Chimpanzee
Rhesus monkey
Dog
Cattle
Horse
Pig
Rabbit
Mouse
Rat

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                5555555555555555555555555555555555555555555555555555555555555555555555555555555555555555666666666666666666666666666666666666666666666666
                2222222223333333333444444444455555555566666666667777777777888888888899999999000000000011111111112222222223333333334444444444555
                1234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890
Human           CSQRGECLCGQCVCHSSDFGKITGKYCECDDFSCVRYKGEMCSGHGQCSCGDCLCDSDWTGYYCNCTTRTDTCMSSNGLLCSGRGKCECGSCVCIQPGSYGDTCEKCPTCPDACTFKKECVECKKFDRGA
Chimpanzee      ..................................................................................................................................
Rhesus monkey...................................................................................................................................
Dog             ...........................................................................G..........................................................T
Cattle          ..................................................................................................................................N...
Horse           ..............T.....................................................................................................................E...
Pig             ....................................H.........................F.........L...........N.....................................G.......N..S
Rabbit          ..................................................................................................................................E..V
Mouse           ..............................................N....V.....................T.........N........V...................S...........N..T
Rat             ..............................................N....V.....................T.........N........V...................S...D.......N..K

                6666666666666666666666666666666666666666666666666677777777777777777777777777777777777777777777777777777777777777777777777777777
                5555555556666666666777777777788888888899999999990000000000111111111222222222233333333334444444455555555556666666666677777777778
                1234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890
Human           LHDENTCNRYCRDEIESVKELKDTGKDAVNCTYKNEDDCVVRFQYYEDSSGKSILYVVEEPECPKGPDILVVLLSVMGAILLIGLAALLIWKLLITIHDRKEFAKFEEERARAKWDTANNPLYKEATSTF
Chimpanzee      ..................................................................................................................................
Rhesus monkey.....................................................................................T............................................................
Dog             ...D...............................................................................T............................................................
Cattle          .NE.............P.....................................................................T............................................................
Horse           .YE....T.............M.....................................A.....................T............................................................
Pig             .YEA.................Q.S..N.....................................................T............................................................
Rabbit          ..E....S......M......R.....................................................F.L............................................................
Mouse           ..E....S.....D..Q....T....N.....................T..RAV.....................T............................................................
Rat             ..E..N...F...D..L....T....N.....................RAV.....................T............................................................

                77777777
                88888888
                12345678
Human           TNITYRGT
Chimpanzee      ........
Rhesus monkey........
Dog             ........
Cattle          ........
Horse           ........
Pig             ........
Rabbit          ........
Mouse           ........
Rat             ........
```

Figure 2. Alignment of integrin β3-subunit for 10 mammalian species. The entire human protein sequence is depicted. Signal peptide is in red. Amino acids under positive selection are in black bold. In red bold are depicted the residues that belong to the RGD-binding pocket, that includes the metal ion coordination sites, SyMBS (residues D184, N241, D243, P245 and E246), MIDAS (residues D145, S147, S149 and D277) and ADMIDAS (residues D152, D153 and M361)[25, 31]. In light green, EGF-1 domain. In light blue, EGF-2 domain. In dark green, EGF-3 domain. In dark blue, EGF-4 domain. In purple, the transmembrane domain. In orange, the cytoplasmic domain. Full stop points (.) represent the same amino acid as in human sequence. Dash marks (-) represent indels. Numbers above the sequences are read vertically from top to bottom. GenBank accession numbers: Human (Homo sapiens), NM_000212.2; Chimpanzee (Pan troglodytes), XM_523684.6; Rhesus monkey (Macaca mulata), XM_001116013.2; Mouse (Mus musculus), NM_016780.2; Rat (Ratus norvegicus), NM_153720.1; Rabbit (Oryctolagus cuniculus), NM_001082066.1; Dog (Canis lupus familiaris), NM_001003162.1; Horse (Equus caballus), NM_001081802.1; Pig (Sus scrofa), NM_214002.1; Cattle (Bos taurus), NM_001206490.2.

Discussion

The β-propeller of αIIb and the I-domain of β3 form the "head" of integrin αIIbβ3. This portion of the integrin interacts with extracellular agonists such as von Willebrand factor (vWF) and fibrinogen molecules. The binding domains map of αIIb, showed that the regions comprised by residues 57-64, 265-284 and 313-332 are potentially involved in fibrinogen-binding (Biris et al. 2003). The four residues that are under positive selection in αIIb-subunit are part of the seven-bladed β-propeller. Residues 119 (150) (threonine), 153 (184) (arginine), 162 (193) (tryptophan) and 339 (370) (alanine) lies in the outer surface of the integrin αIIbβ3 in its compact, low affinity conformation (figure 3a and 3b) and in its open, high affinity conformation (figure 3c and 3d). Both 153 (184) and 162 (193) are near to residue 160 (191) (phenylalanine) that is part of the arginine-glycine-aspartic acid (RGD) binding-pocket (Xiong et al. 2001, Biris et al. 2003, Xiong et al. 2003, Ma et al. 2007, Mitchell et al. 2007, Zhu et al. 2010, Choi et al. 2013, Zhu et al. 2013, Coller 2015).

From the three positively selected codons of integrin β3-subunit, 129 (155) is in the I-domain. I-domain contains multiple metal ion coordination sites, identified as MIDAS, ADMIDAS and SyMBS. When β3 associates with αIIb to form integrin αIIbβ3, these sites are important to coordinate, regulate and stabilize the binding of ligands such as fibrinogen and vWF (Xiong et al. 2003). Positions 440 (466) and 444 (470) are localized in EGF-1, a site that is rich in cysteines and contains important structural disulphide bonds (Xiong et al. 2002, Mor-Cohen et al. 2008, Mor-Cohen et al. 2012). Functionally, these EGF domains are important for platelet activation. Integrin αIIbβ3 is inactive in resting platelets. Following platelet activation by agonists like adenosine diphosphate (ADP) or thrombin, it undergoes a conformational change regulated by inside-out signalling. Cysteine residues and their disulphide bonds within EGF-like domains are crucial to regulate this protein structural change (Mor-Cohen et al. 2008, Mor-Cohen et al. 2012). After this conformational step, integrin αIIbβ3 is now able to bind fibrinogen and vWF through its globular head that comprises the β-propeller of αIIb-subunit and the I-domain from β3-subunit. Residues 129 (155) (tryptophan), 440 (466)

(glutamine) and 444 (470) (asparagine) all lies in the outer surface of the integrin in its compact, low affinity conformation and when the integrin is in its open, high affinity conformation and bound to a RGD motif (see figures 3a, 3b, 3c and 3d). Residue 129 (155) is extremely close to residues 126 (152) and 127 (153) (both aspartic acids) that are part of ADMIDAS site included in the RGD binding-pocket. Figure 4 shows that residues 119 (150), 153 (184) and 162 (193) from integrin αIIb-subunit and residue 129 (155) from integrin β3-subunit cluster around the RGD binding pocket, depicted in green in the figure. Although the literature does not refer exactly the function of any of the residues identified here as evolving under positive selection, it seems likely that some kind of pressure generate the amino acid changes that we describe here. One hypothesis that might explain why these specific codons are suffering positive selection is the presence of external factors that could use integrin αIIbβ3 for some specific purpose. Interestingly, Andes virus, responsible for hantavirus pulmonary syndrome in humans and Syrian hamsters, recognize a specific Leucine to Proline substitution in position 33 in the PSI domain of β3-subunit of integrin αVβ3 from endothelial cells to infect those cells (Matthys et al. 2010). In this study, we found that three of the residues that are under positive selection among mammalian species, show, also, SNP's in humans, two of them being non-synonymous polymorphisms. In our particular case, the affected residues (153 (184) from αIIb-subunit and 129 (155) from β3-subunit) did not seem to have any previously known clinical significance. Nonetheless, it is acknowledged that SNP's in platelet membrane receptors may influence platelet adhesion and aggregation (Afshar-Kharghan et al. 2007). These two polymorphic residues are part of the four residues that are under positive selection and cluster around the RGD-binding pocket (figure 4), suggesting that this particular site of the integrin is prone to some kind of exterior pressure which leads to changes in the residues. Besides the amino acid substitutions that, per se, may probably change the avidity of potential pathogens to the protein, there are also physicochemical differences in each one of the seven positively selected positions, what may impose changes in the region of the protein where these amino acids lay (see tables I and II). The presence of positively selected codons among mammals may suggest a host

adaptation to increase the genetic resistance against pathogenicity and virulence. Additional functional and structural studies will help to elucidate why this positive selection occurs in these specific residues.

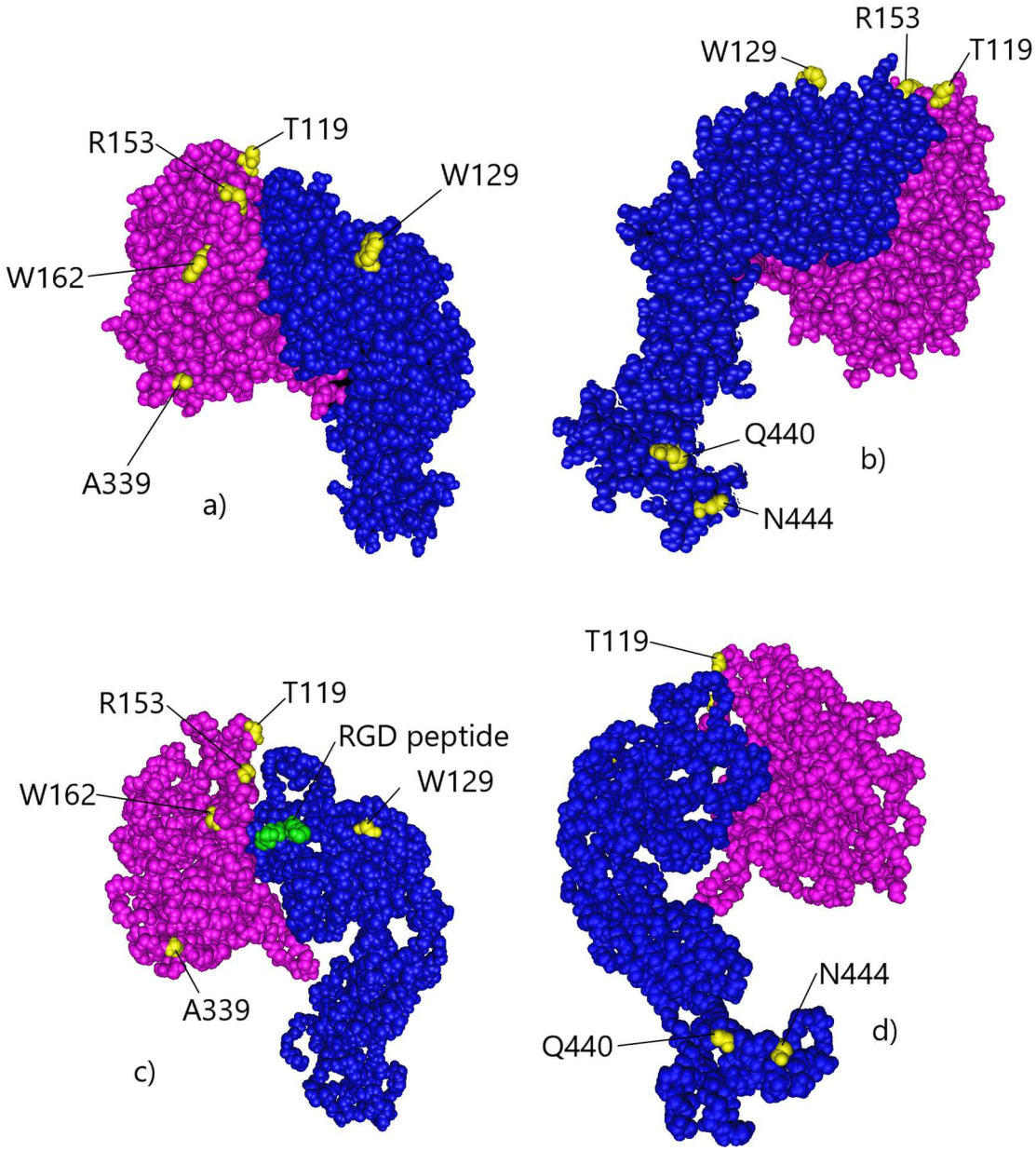

Figure 3. a and b) 3D structure of integrin αIIbβ3 headpiece in its closed, low affinity state. αIIb-subunit is in pink and β3-subunit is in blue. Residues highlighted in yellow are those identified to be under positive selection. **a)** Residues T119, R153, W162 and A339 from αIIb-subunit and residue W129 from β3-subunit are shown Not shown are residues Q440 and N444 from β3-subunit that lies in the opposite part of the integrin. **b)** Opposite part of the integrin where residues T119 and R153 from αIIb-subunit and residues W129, Q440 and N444 from β3-subunit are shown (Retrieved from NCBI Structure. PDB ID: 3NID. MMDB ID: 87460). **c and d)** 3D structure of integrin αIIbβ3 headpiece in its open, high affinity state. αIIb-subunit is in pink and β3-subunit is in blue. Residues highlighted in yellow are those identified to be under positive selection. **c)** Residues T119, R153, W162 and A339 from αIIb-subunit and residue W129 from β3-subunit are shown. In green appears a RGD peptide (GRGDSP) bound to the RGD binding-pocket. Not shown are residues Q440 and N444 from β3-subunit that lies in the opposite part of the integrin. **d)** Opposite part of the integrin where residue T119 from αIIb-subunit and residues Q440 and N444 from β3-subunit are shown. (Retrieved from NCBI Structure. PDB ID: 3ZE2. MMDB ID: 110629).

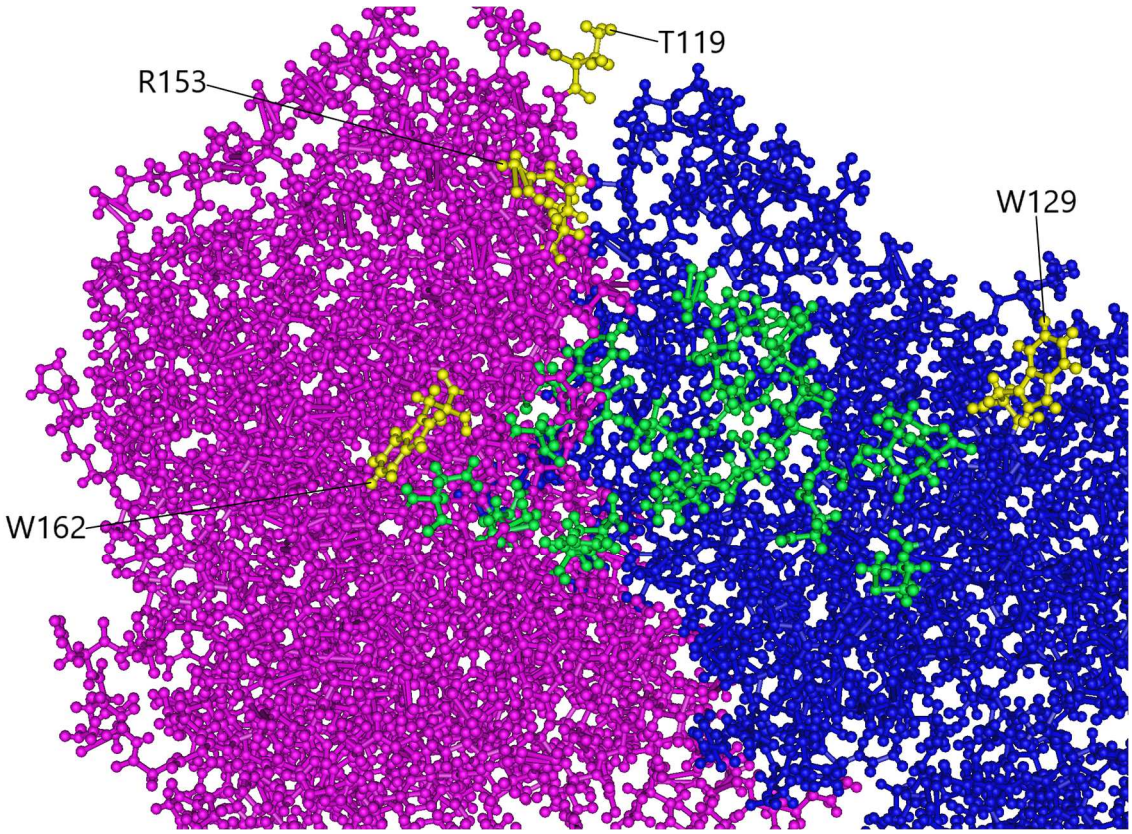

Figure 4) Detailed section of integrin αIIbβ3 headpiece in its open, high affinity state showing four of the seven residues that are under positive selection clustering around the RGD -binding pocket. αIIb-subunit is in pink and β3-subunit is in blue. Residues T119, R153 and W162 from αIIb-subunit and residue W129 from β3-subunit are highlighted in yellow. In green are depicted the residues that belong to the RGD-binding pocket (residues F160, Y190, L192, D224, F231 and D232 from integrin αIIb-subunit and residues D119, S121, S122, S123, D126, D127, D158, R214, N215, D217, A218, P219, E220, D251 and M335 from integrin β3-subunit [25, 31]). Retrieved from NCBI Structure. PDB ID: 3ZE2. MMDB ID: 110629.

Conflict of interest statement

The authors declare no conflict of interest.

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Capítulo 5: Discussão geral

Importância das alterações genéticas das plaquetas

As plaquetas sanguíneas são elementos essenciais na prossecução normal de todos os eventos vasculares, celulares e moleculares envolvidos na hemóstase. Para conseguirem desempenhar as suas funções, as plaquetas sanguíneas exibem uma quantidade de recetores capazes de integrar informação veiculada por fatores externos que sinalizam o estado do vaso sanguíneo por onde a plaqueta está a circular. Desta forma, em situações fisiológicas normais, as plaquetas circulam sem aderir às paredes do vaso ou umas às outras no vaso sanguíneo íntegro. No entanto, são capazes de alterar o seu comportamento nas zonas vasculares lesadas e aderir à matriz subendotelial exposta, formando um agregado plaquetário que limita a perda sanguínea no local da rutura vascular.

Além da sua participação na hemóstase, é igualmente reconhecido o seu importante papel fisiológico em eventos como a fibrinólise (Whyte et al. 2017), a regeneração de tecidos (Etulain 2018), a inflamação (Bergmeier and Wagner 2007), a sinalização de leucócitos (Nording and Langer 2018) e a angiogénese (Brill and Varon 2007). Da mesma forma que participam nos mecanismos fisiológicos descritos, as plaquetas também estão envolvidas em eventos patológicos como a progressão tumoral, a metastização e a trombose (Savage and Ruggeri 2007, Meikle et al. 2016). Quer seja nos mecanismos fisiológicos ligados à manutenção da homeostasia vascular, quer seja nos processos patológicos que eventualmente terminam em eventos catastróficos como o enfarte agudo do miocárdio, a interação entre as plaquetas e o meio circundante faz-se através de recetores plaquetários.

Recetores plaquetários como o recetor da prostaciclina ou o recetor para o NO (sGC), responsáveis pela manutenção do estado quiescente das plaquetas na vasculatura íntegra, ou recetores implicados nos processos de ativação plaquetária, como a GPIb-IX-V e a integrina $\alpha 2b\beta 3$, têm sido amplamente estudados devido à sua importância, seja na função plaquetária normal, seja na participação das plaquetas em eventos fisiopatológicos como a trombose. Em termos clínicos, é importante realçar o desenvolvimento de fármacos que atuam nestes recetores com o intuito de reduzir a capacidade

agregante das plaquetas sanguíneas em doentes com uma maior propensão pró-trombótica. Desta forma, qualquer alteração genética ligada às proteínas plaquetárias pode determinar uma resposta diferente consoante o estímulo presente no meio.

Os estudos relacionados com os polimorfismos das proteínas plaquetárias podem ajudar quer na compreensão do papel fisiológico dessas proteínas nos mecanismos normais da hemóstase, quer na sua relação com determinadas doenças cardiovasculares em que as plaquetas estão implicadas. Assim, está descrita a participação de variantes plaquetárias polimórficas em fenómenos trombóticos como o enfarte agudo do miocárdio (Santoso et al. 1999, Casorelli et al. 2001) ou o acidente vascular cerebral (Liu et al. 2017). Da mesma forma, a resposta destes doentes aos fármacos administrados também poderá variar consoante as variações genéticas plaquetárias intrínsecas do doente (Ulehlova et al. 2014). No entanto, muitos dos resultados anteriormente publicados são contraditórios entre si, expondo desta forma uma característica intrínseca das doenças trombóticas cardiovasculares, a sua génese multifatorial e poligénica.

Diversos fatores de risco ambientais estão descritos como potenciadores do aparecimento e desenvolvimento da patologia cardiovascular (Cosselman et al. 2015). Da mesma forma, algumas alterações genéticas polimórficas já são utilizadas como marcadores de maior risco para o desenvolvimento de doenças cardiovasculares, como é o caso da hiperhomocisteinemia devido à presença de polimorfismos na enzima metilenotetrahidrofolato redutase (MTHFR) (Cortese and Motti 2001) ou a hipercolesterolemia familiar em que se encontram alterações genéticas nos recetores das lipoproteínas de baixa densidade (LDL) (Defesche et al. 2017).

Do ponto de vista clínico, para tratar este tipo de doentes é preciso ter em atenção a sua resposta aos fármacos atualmente em uso, sabendo-se, mais uma vez, que determinados polimorfismos poderão alterar a resposta ao fármaco ou à sua dose (Zhou et al. 2009, Shukla et al. 2018).

Do exposto anteriormente, fica patente a necessidade de estudar os mecanismos moleculares e genéticos que podem conferir um risco aumentado de aparecimento e desenvolvimento de patologias cardiovasculares, assim como influenciar a resposta às terapias anti-trombóticas.

Resultados principais em relação aos objetivos específicos do trabalho

Objetivo I. Descrição das frequências alélicas e genotípicas dos polimorfismos de recetores plaquetários e da eNOS na população portuguesa.

Uma vez que não existiam dados publicados relativos às frequências alélicas e genotípicas das glicoproteínas plaquetárias mais relevantes para a função das plaquetas, foi realizado um primeiro estudo onde caracterizou-se a população portuguesa em relação aos principais polimorfismos das glicoproteínas GPIIIa, GPIbα e GPIa (resultados incluídos no capítulo 2 da tese). Neste trabalho verificou-se que a população portuguesa se encontrava em equilíbrio de *Hardy-Weinberg* em relação aos polimorfismos estudados e que não existiam diferenças significativas entre as frequências alélicas e genotípicas obtidas e as encontradas em outras populações de origem caucasiano. Para além deste trabalho, outros polimorfismos de proteínas importantes na função plaquetária, nomeadamente da enzima eNOS e do recetor do ADP $P2Y_{12}$, também foram estudados em termos de frequências alélicas e genotípicas na população portuguesa (resultados incluídos no capítulo 3). Assim, os resultados aportados por este trabalho são importantes dado que a obtenção destas frequências na população portuguesa poderá ser usada em estudos subsequentes que eventualmente incidam sobre a relação destes polimorfismos e a função plaquetária ou qualquer doença em que as plaquetas participem.

Das frequências obtidas para os diferentes polimorfismos estudados, destacamos as encontradas na glicoproteína GPIbα:

I. A frequência alélica do alelo T do polimorfismo rs6065 (HPA-2), 9,4%, e a ausência de qualquer indivíduo com o genótipo TT;

II. A frequência do alelo C do polimorfismo rs2243093 (Kozak), 10,5%, e a deteção de apenas 1 indivíduo com o genótipo CC;
III. A baixa frequência detetada do alelo E do polimorfismo VNTR, 0,2%, e a deteção de apenas 1 indivíduo com o alelo E (genótipo CE). Assim como a ausência na população estudada do alelo A do polimorfismo VNTR.

Objetivos II. e III. Análise das frequências alélicas e genotípicas dos polimorfismos estudados em doentes que sofreram enfarte agudo do miocárdio e as suas possíveis implicações clínicas.

O corpo central deste trabalho teve por objetivo específico o estudo da possível associação entre os polimorfismos estudados e o enfarte agudo do miocárdio. Para atingir este objetivo, avaliámos o possível efeito dos polimorfismos em doentes que sofreram enfarte agudo de miocárdio. Estes doentes foram divididos em dois grupos, um formado por doentes que sofreram um primeiro episódio de enfarte agudo de miocárdio com 45 anos ou antes, e outro constituído por doentes que sofreram o primeiro episódio depois dos 45 anos (resultados incluídos no capítulo 3 da tese).

Dos resultados obtidos, em relação aos polimorfismos das glicoproteínas plaquetárias, podemos destacar:

I. Ausência de associação significativa entre os polimorfismos estudados e o aparecimento e desenvolvimento de enfarte agudo do miocárdio em doentes com menos de 45 anos de idade;
II. Associação significativa do genótipo TT do polimorfismo rs938043469 (807C>T) do gene *ITGA2*, responsável por codificar a subunidade $\alpha 2$ do recetor plaquetário para o colagénio $\alpha 2\beta 1$ (GPIa), e o enfarte agudo do miocárdio em doentes com mais de 45 anos de idade.

Em relação aos polimorfismos da enzima eNOS, dos resultados obtidos, podemos destacar:

I. Ausência de associação significativa entre os polimorfismos estudados e o aparecimento e desenvolvimento de enfarte agudo do miocárdio em doentes com mais de 45 anos de idade;
II. Associação significativa do genótipo TC do polimorfismo rs2070744 (-786T>C) do gene *NOS3* e o enfarte agudo do miocárdio em doentes com menos de 45 anos de idade;
III. Um aumento (embora não significativo, conforme se descreve no capítulo 3) de doentes com genótipo TT do polimorfismo rs1799983, no grupo de doentes com menos de 45 anos, em relação à população normal (29,5% *vs.* 13,9%).

Embora se tenha de ter em consideração as inerentes limitações do trabalho (referidas nos trabalhos 2 e 3 do capítulo 3 da tese), dos resultados obtidos podemos concluir que os polimorfismos das glicoproteinas plaquetárias, assim como da eNOS, podem ter um importante papel na doença trombótica. No entanto o seu envolvimento nos mecanismos fisiopatológicos parece ser diferente: os recetores plaquetários, não parecem ter uma influência no aparecimento precoce do enfarte agudo de miocárdio, uma vez que a análise estatística mostrou que o polimorfismo rs938043469 da GPIa apresenta uma associação significativa com a doença, mas apenas em doentes com mais de 45 anos. Contrariamente, os polimorfismos da enzima eNOS parecem ter uma maior influência no início precoce da doença, uma vez que a análise estatística revelou a sua associação com a doença, mas apenas em doentes com menos de 45 anos.

Objetivo IV. Estudo evolutivo de recetores plaquetários: comparação entre 10 sequências nucleotídicas de mamíferos referentes à integrina α2bβ3.

Sabendo que a variação genética das proteínas plaquetárias entre diferentes espécies pode revelar aspetos importantes relacionados com a função da proteína, foi realizado o estudo das variantes genéticas da integrina α2bβ3 (GPIIb-IIIa), com o intuito de saber se apresentava alguma alteração significativa na sua estrutura entre diferentes espécies de mamíferos. Pretendíamos avaliar se durante a evolução este fulcral recetor plaquetário sofreu qualquer tipo de pressão externa que levasse ao aparecimento de modificações com o intuito de se adaptar a essa pressão. Assim, no capítulo 4, estão descritas as principais alterações encontradas, das quais realçamos:

I. A deteção de 4 resíduos da subunidade α2b e 3 na subunidade β3 que apresentam diferenças entre as várias espécies de mamíferos estudadas, o que pressupõe que nesses locais está a atuar uma pressão externa que obriga a proteína a adaptar-se, alterando a sua estrutura.
II. A localização de 4 desses resíduos muito próximos dos resíduos que formam o local de ligação dos principais agonistas da integrina α2bβ3, como por exemplo, o fibrinogénio.
III. A existência, nos humanos, de polimorfismos previamente descritos em pelo menos 3 dos 7 resíduos sob pressão seletiva, sendo que 2 desses polimorfismos são substituições não-sinonímicas que implicam alteração de aminoácidos, realçando desta forma a existência de pressão externa que obriga a diversificação destes aminoácidos.

Conclusão

Como conclusão do trabalho podemos dizer que os resultados obtidos revelam a importância do estudo dos recetores plaquetários e da eNOS, assim como da sua participação na doença trombótica, particularmente no enfarte

agudo de miocárdio. São necessários estudos funcionais e clínicos para elucidar o papel que as variantes genéticas destas proteínas poderão ter no estabelecimento de um perfil genético individual que contribua para uma adequada estratificação dos doentes e para o desenho e desenvolvimento de novas estratégias terapêuticas e esquemas terapêuticos antiplaquetários baseados na interindividualidade.

Estudos futuros

O estudo do papel das variantes genéticas plaquetárias nas doenças cardiovasculares continua a merecer atenção, principalmente devido ao número crescente de alterações detetadas em diferentes populações (Sevivas et al. 2018). Os novos avanços tecnológicos, nomeadamente a sequenciação de nova geração (NGS) e as ferramentas bioinformáticas, têm permitido detetar mais rapidamente alterações genéticas simples, o que leva a um aumento significativo do conhecimento do número de polimorfismos conhecidos numa determinada população. Muitas destas alterações genéticas não apresentam nenhum significado clínico conhecido, no entanto podem estar envolvidas no aumento da suscetibilidade a vários níveis e fazer aumentar ou diminuir a predisposição para o aparecimento e desenvolvimento de doenças complexas associadas a alterações em múltiplos genes, como é o caso das doenças cardiovasculares. Se a reatividade plaquetária depender em grande parte das alterações polimórficas exibidas pelos seus recetores (Strisciuglio et al. 2015), o estudo dessas alterações torna-se um tópico importante, principalmente aplicado ao tratamento antiplaquetário usado após um evento trombótico arterial (Sibbing et al. 2011). Assim, sempre que um novo fármaco, com efeito sobre as plaquetas, entra em uso, é necessário perceber como é que as variantes polimórficas plaquetárias vão reagir perante esse novo fármaco. Para analisar a reatividade plaquetária, vários métodos funcionais podem ser usados, como o teste *Platelet Function Analizer 100* (PFA-100®), *VerifyNow*®, citometria de fluxo e *Multiplate*® (Danielak et al. 2017). Assim, uma linha de trabalho futuro prende-se com a análise funcional das plaquetas dependendo dos polimorfismos estudados. Como foi referido no artigo 1 do capítulo 3

(Pina-Cabral et al. 2018), realizámos estudos funcionais preliminares usando o PFA-100® tendo em consideração o polimorfismo VNTR e o diferente tamanho da subunidade GPIb da GPIb-IX-V (resultados não publicados). Os resultados obtidos pelo PFA-100 para os diferentes polimorfismos estudados estão a ser compilados e poderão proporcionar evidências relativamente às diferenças na reatividade plaquetária medida pelo PFA-100®, úteis no estudo do efeito de fármacos dirigidos para a função plaquetária.

Estudos futuros com um maior número de doentes e grupos mais homogéneos em termos de variáveis não genéticas seriam importantes para esclarecer outra das questões que a nosso ver ficou em aberto neste trabalho e que se prende com a relação do genótipo TT do polimorfismo rs1799983 ligado à eNOS, no enfarte agudo do miocárdio nos doentes com menos de 45 anos. Uma vez que os resultados foram muito próximos da significância estatística, seria importante verificar se o resultado se alteraria aumentando o *n* da amostra de doentes.

Para além destas questões, também será importante no futuro esclarecer a possível associação entre os polimorfismos estudados e o resultado clínico dos grupos de doentes. Estes estudos poderiam contribuir para o estabelecimento de uma estratificação mais adequada dos doentes com enfarte agudo de miocárdio, em função do seu perfil genético.

Para além dos aspetos referidos, os resultados obtidos relativos à deteção de locais sob seleção positiva para a integrina $\alpha 2b\beta 3$ abre várias hipóteses de estudo. Uma das hipóteses seria tentar perceber quais os fatores que impõem variabilidade entre as espécies estudadas, sabendo de antemão que já estão descritas situações em que a alteração de um aminoácido na estrutura do recetor $\alpha v\beta 3$ permite uma maior ou menor patogenicidade do fator vírico. Outra hipótese seria perceber se os polimorfismos que existem nos locais sob seleção implicam alterações na reatividade plaquetária. Ao mesmo tempo, o trabalho que foi feito para a integrina $\alpha 2b\beta 3$ poderá ser aplicado aos outros recetores plaquetários de interesse fisiológico.

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Anexos

Artigo 1

Associação entre o ligando CD40 solúvel na progressão do enfarte agudo do miocárdio e polimorfismos da eNOS, fator de crescimento endotelial e expressão de CD62P plaquetário

Changes of soluble CD40 ligand in the progression of acute myocardial infarction associate to endothelial nitric oxide synthase polymorphisms and vascular endothelial growth factor but not to platelet CD62P expression

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Reported in vitro data implicated soluble CD40 ligand (sCD40L) in endothelial dysfunction and angiogenesis. However, whether sCD40L could exert that influence in endothelial dysfunction and angiogenesis after injury in acute myocardial infarction (AMI) patients remains unclear. In the present study, we evaluated the association of sCD40L with markers of platelet activation, endothelial, and vascular function during a recovery period early after AMI. To achieve this goal, the time changes of soluble, platelet-bound, and microparticle-bound CD40L levels over 1 month were assessed in AMI patients and correlated with endothelial nitric oxide synthase (eNOS) polymorphisms, vascular endothelial growth factor (VEGF) concentrations, and platelet expression of P-selectin (CD62P). The association of soluble form, platelet-bound, and microparticle-bound CD40L with CD62P expression on platelets, a marker of platelet activation, was also assessed to evaluate the role of CD40L in the thrombosis, whereas the association with eNOS and VEGF was to evaluate the role of CD40L in vascular dysfunction. This work shows for the first time that time changes of sCD40L over 1 month after myocardial infarct onset were associated with G894T eNOS polymorphism and with the VEGF concentrations, but not to the platelet CD62P expression. These results indicate that, in terms of AMI pathophysiology, the sCD40L cannot be consider just as being involved in thrombosis and inflammation but also as having a relevant role in vascular and endothelial dysfunction. (Translational Research 2015;166:650–659)

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Abbreviations: AMI = acute myocardial infarction; APC = allophycocyanin; Asp = aspartate; cTNT = cardiac troponin; CAD = coronary artery disease; CRP = C-reactive protein; CK = creatine kinase; eNOS = endothelial nitric oxide synthase; ELISA = enzyme-linked immunosorbent assay; FITC = fluorescein isothiocyanate; FAU = fluorescence arbitrary units; Glu = glutamic acid; LME = linear mixed effects model; MPs = microparticles; NO = nitric oxide; NT-proBNP = N-terminal pro-brain natriuretic peptide; PCI = percutaneous coronary intervention; PBS = phosphate-buffered saline; PE = Phycoerythrin; CD62P = P-selectin; sCD40L = soluble CD40 ligand; SA = stable angina pectoris; VEGF = vascular endothelial growth factor

AT A GLANCE COMMENTARY

Napoleão P, et al.

Background

The influence of sCD40L in endothelial dysfunction and angiogenesis markers in AMI patients remains unclear. The influence of sCD40L in endothelial and vascular function in early stages after AMI was studied having platelet activation into account.

Translational Significance

It was found for the first time that time changes of sCD40L in AMI patients were associated with G894T eNOS polymorphism and VEGF concentrations, but not to the platelet P-selectin expression. In terms of AMI pathophysiology, sCD40L cannot be considered just as a marker of thrombosis and inflammation but has also a relevant role in vascular and endothelial dysfunction. A prognostic value for the sCD40L could be hypothesized along the progression of the disease in AMI patients. This may be of the utmost importance in clinical diagnostic of AMI.

INTRODUCTION

CD40L is a signaling molecule,[1-3] implicated in thrombosis and inflammatory response to vascular injury.[4-6] The relationship of CD40L with coronary artery disease (CAD) has been established,[2,7-9] as also its implication in endothelial dysfunction.[10-14] However, whether the soluble CD40 ligand (sCD40L) could also influence endothelial dysfunction after acute myocardial infarction (AMI) injury remains unclear.

In vitro studies have shown that sCD40L inhibits angiogenesis and also growth factor–induced human umbilical vein endothelial cell migration, which is achieved by generation of free radicals and inhibition of nitric oxide (NO) production.[10] The authors hypothesized that the sCD40L could inhibit reendothelialization of an injured vessel, thereby affecting the restenosis.[10]

Research efforts have been directed toward the finding of biomarkers to assess endothelial function and its correlation with AMI. Genetic indicators, such as the polymorphisms of endothelial NO synthase (eNOS) gene,[15,16] may provide insight into endothelial cells function.

Vascular endothelial growth factor (VEGF) is a well-known promoter of angiogenesis and an endogenous regulator of endothelial integrity.[17-19] The prognostic information provided by VEGF independently of other markers likely points toward an important role for angiogenesis in regulating myocardial repair and reperfusion after AMI.[17,20]

Current opinion suggests a differential role of CD40L (both soluble and membrane-bound forms, which includes microparticles in circulation)[21] at different stages of CAD, contrasting with the traditional view of an unvarying function of the CD40L-CD40-sCD40L system interactions in the disease.[6] In that perspective, no clear indication of the interplay of CD40L with endothelial and vascular function markers and their importance in the pathophysiology of the AMI has been obtained so far in human clinical studies. Therefore, the aim of this study was to evaluate the relationship of sCD40L with markers of platelet activation, endothelial and vascular function during an early recovery period after AMI. To achieve this goal, the time changes over 1 month of sCD40L levels were assessed in AMI patients and correlated with the CD40L expressed on platelets and microparticles, CD62P expression on platelets, and eNOS polymorphisms VEGF concentrations. The association of soluble form, platelet-bound, and microparticle-bound CD40L with CD62P expression on platelets was assessed to evaluate the role of CD40L in thrombosis, whereas the association with eNOS and VEGF was to evaluate the role of CD40L in vascular dysfunction. The sCD40L serum concentrations were measured and compared with the expression of CD40L on platelets and microparticles. Healthy volunteers (CTR) and longitudinally assessed stable angina (SA) patients were used as predictors of altered endothelial regulation in AMI.

Table I. Baseline demographic and clinical characteristics of the study population

	CTR (n = 63)	SA (n = 48)	AMI (n = 89)
Sex (f/m)	23/40	13/35	21/67
Age (y)	55 (47–65)	63 (57–73)	63 (54–72)
BMI (kg/m^2)	25 (24–28)	28 (25–29)	27 (24–30)
Waist perimeter (cm)	86 (82–94)	96 (91–102)	99 (89–106)
Genotyping polymorphisms			
eNOS G894T (GG/T), %	38/62	37/63	38/63
eNOS T786C (TT/C), %	43/57	50/50	28/72*
Risk factors and comorbidity			
Hypertension, n (%)	18 (29)	37 (77)†	58 (65)†
Hyperlipidemia, n (%)	29 (46)	35 (73)†	47 (53)*
Diabetes, n (%)	2 (3)	16 (33)†	35 (39)†
Family history of CAD, n (%)	5 (8)	12 (25)†	8 (9)
Smoking, n (%)	4 (6)	4 (8)	39 (44)*,†
Medication			
Pre-event medication			
Without previous treatment, n (%)	46 (73)	5 (10)†	27 (30)*,†
Aspirin, n (%)	4 (6)	32 (67)†	41 (46)*,†
ACE inhibitors, n (%)	9 (14)	24 (50)†	30 (34)†
β blockers, n (%)	3 (5)	22 (46)†	26 (24)*,†
Statins, n (%)	10 (16)	37 (77)†	35 (39)*,†
Postevent medication			
Aspirin, n (%)	—	28 (58)	77 (87)
ACE inhibitors n (%)	—	12 (25)	64 (72)*
GP IIb/IIIa inhibitors, n (%)	—	24 (50)	78 (88)*
ADP-receptor inhibitors, n (%)	—	4 (8)	44 (49)*
β blockers, n (%)	—	8 (17)	60 (67)*
Statins, n (%)	—	26 (54)	72 (81)

Abbreviations: ACE, angiotensin-converting enzyme; ADP, adenosine diphosphate; AMI, acute myocardial infarction; BMI, body mass index; CAD, coronary artery disease; GP IIb/IIIa, glycoprotein IIb/IIIa; SA, stable angina.
Data expressed as median and interquartiles (Q25–Q75), except when otherwise indicated.
*$P < 0.05$ vs SA group.
†$P < 0.05$ vs CTR group.

MATERIALS AND METHODS

Study groups. A total of 200 subjects were recruited from cardiology service and from the outpatient clinic of cardiovascular risk in Santa Marta Hospital (Lisbon, Portugal; Table I): (1) 89 AMI patients (with documented ST-elevation changes, creatine kinase [CK] >3 times normal and troponin positive) undergoing percutaneous coronary intervention (PCI) as reperfusion therapy were enrolled during the first 6 hours of the onset of chest pain (hospital admission); (2) 48 age- and sex-matched patients with SA pectoris, presenting typical chest discomfort and/or positive stress tests, which were submitted to coronary angiography; and (3) 63 healthy volunteers (CTR), with negative stress test, absence of any history of coronary disease, life-threatening diseases, or any other disease or condition that would impair compliance. These volunteers were not submitted to coronary angiography.

Patients' exclusion criteria included age >85 years; significant comorbidities as peripheral artery disease or carotid artery disease; known antecedents of malignant, infectious, and concurrent inflammatory diseases; chronic renal insufficiency; and previous myocardial infarction during the previous 5 years.

Core laboratory blood analysis for conventional tests and clinical chemistry, including N-terminal pro-brain natriuretic peptide, C-reactive protein, CK, and cardiac troponin were measured in all patients and controls (Table II).

Study protocol and blood sampling. The AMI and SA patients were monitored at 2 time points: day 0 before PCI intervention and the administration of therapy, such as antithrombotic agents and IIb/IIIa inhibitors; 1 month after PCI. This protocol was designed to evaluate AMI patients at the acute phase of AMI (rupture and coronary occlusion) and at the early recovery phase (left ventricular remodeling), 1 month after. Previous studies indicated that the influence of medication in the values of inflammatory markers lasted for several days after PCI.[22,23] Therefore, patients' assessment at 1-month evaluation represents the period of time for medication and clinical stabilization.

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Table II. Biochemical data in the studied population groups

		SA		AMI	
	CTR	Admission	1 month	Admission	1 month
Inflammatory markers					
CRP (mg/dL)	3.1 (1.2–3.5)	3.2 (1.7–5.9)	1.4 (1.1–4.1)	6.3 (3.2–12)	3.2* (3.1–6.7)
sCD40L (ng/mL)	4.1 (2.2–6.5)	1.1† (0.68–2.2)	5.6†,‡ (3.0–8.5)	1.3† (0.73–3.4)	2.0*,† (0.79–3.3)
Vascular function marker					
VEGF (pg/mL)	419 (212–758)	18† (1.1–295)	293† (192–442)	48† (0.27–266)	275* (161–493)
Cardiac function markers					
CK (U/L)	111 (80–195)	82 (58–107)	97 (35–159)	315†,§ (113–1062)	79* (65–118)
cTnT (ng/mL)	<0.01‖	<0.01‖	<0.01‖	0.33†,§ (0.07–2.4)	<0.01‖
NT-proBNP (pg/mL)	38 (16–64)	98 (51–247)	155 (55–424)	356†,§ (145–1577)	637* (618–1404)

Abbreviations: AST, aspartate transaminase; AMI, acute myocardial infarction; cTnT, cardiac troponin T; CRP, C-reactive protein; CK, creatine kinase; NT-proBNP, N-terminus pro-B-type natriuretic peptide; SA, stable angina; sCD40L, soluble CD40 ligand; VEGF, vascular endothelial growth factor.
Data are expressed as median and quartiles (lower 25% quartile–upper 75% quartile). N.D.
*$P < 0.05$ LME for AMI group variations over time.
†$P < 0.05$ vs Control group.
‡$P < 0.05$ LME for SA group variations over time.
§$P < 0.05$ vs SA group at hospital admission.
‖Values below detection limit.

The study was conducted according to the principles expressed in the Declaration of Helsinki. The Ethical Committee Board of Centro Hospitalar de Lisboa Central approved the investigation and the protocol. All patients and volunteers enrolled signed a written consent following a full explanation of the study.

Blood samples were drawn into blood collection tubes without additives. For AMI and SA patients at hospital admission, the blood was collected immediately before PCI. For the following time point and for healthy volunteers (CTR), fasting blood samples were collected in early morning to avoid possible circadian variations.[24]

The serum was collected after centrifugation (500 g for 10 minutes) within 15 minutes after sampling. Aliquots were stored at −80°C until further analysis (no longer than 6 months). Samples were thawed only once.

Soluble CD40L determination. Concentrations of sCD40L were measured in serum by enzyme-linked immunosorbent assays (ELISA) commercial kit (R&D Systems). Each sample was measured in duplicate. The intra-assay variation among the duplicates for all samples was <10%, and concentrations were expressed in ng/mL.

The choice of serum to determine sCD40L by ELISA in this study had into consideration published data,[25,26] and exploratory analysis performed by us. Ahn et al[25] demonstrated that as long as preanalytical conditions were kept <4°C neither blood origin (venous and arterial) nor blood fraction could significantly modify analytical results. However, Varo et al[26] reported higher sCD40L concentrations in serum than those in plasma. One of the sources contributing to higher serum levels is platelets, therefore, poor-platelet plasma is recommended.[25]

We have performed sCD40L determinations on random blood samples (n = 42) on both serum and poor-platelet plasma fractions. For almost 40% of the analyzed samples (n = 15), sCD40L concentrations in poor-platelet plasma were below the detection limit of the ELISA assay, contrasting with none in serum. Therefore, in the present study, the blood fraction used was serum. To safeguard sCD40L, stability temperature was kept at 4°C in all steps after blood collection, that is, transport and processing.

Platelet activation. Platelet activation was assessed by flow cytometry in whole blood samples within 3 hours after sampling as described previously.[27] Blood was drawn into sodium-citrated blood collection tubes under minimal tourniquet pressure to avoid artifact platelet activation. A fluorescein isothiocyanate-conjugate anti-CD42a antibody (BD) was used as an activation-independent marker of platelets. The expression of P-selectin was assessed by allophycocyanin-conjugate anti-CD62P antibody (BD). Platelets were identified on the basis of size and of fluorescein isothiocyanate-CD42a binding.

Microparticles identification. Microparticles were identified using an adjustment to the method previously described by Bernal-Mizrachi et al.[28] In brief, platelet-poor plasma, obtained by double whole blood centrifugation, was incubated with fluorochrome-labeled antibodies, phosphate-buffered saline (PBS) was added, and the sample was set to flow cytometry. Microparticles were identified on the basis of size defined by scattered light properties using platelets as an internal individual size standard in each sample (Fig. 1).[29] The flow cytometer calibration and the

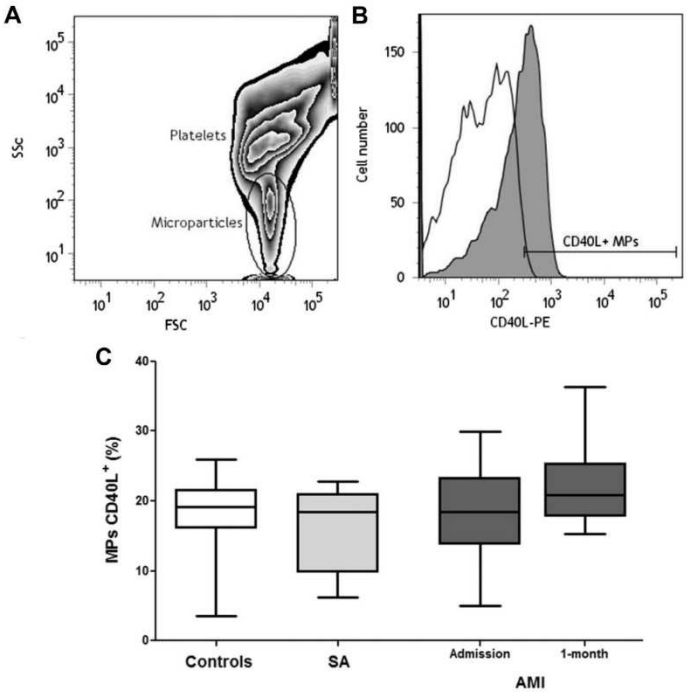

Fig 1. Identification of microparticles. **(A)** Representative flow cytometry zebra plot (contour and density) showing the region within elliptic area "Microparticles" localized below "Platelets," defined by scattered light properties (FSC vs SSC). **(B)** Representative flow cytometry fluorescence histogram showing the surface CD40L-positive microparticles gated on microparticles region. **(C)** Box-and-whisker plot of the percentage of CD40L-positive microparticles for controls, SA, and AMI patients. Plots depict the dispersion of the numeric values (box, 25%–75% interquartile; horizontal line, median; whiskers, minimum and maximum values). AMI, acute myocardial infarction; FSC, forward scattered light; MPs, microparticles; PE, phycoerythrin; SA, stable angina; SSC, side scattered light.

microparticles gate were set using polystyrene microspheres standards (Bangs Laboratories, Inc.) of 0.4 μm and 0.69 μm of nominal diameter.[30,31] The microparticles gate was positioned below platelets, which were then used in each sample as a size internal control. Only the events falling in this gate were analyzed for fluorescence.

CD40L expression on platelets and microparticles. CD40L surface expression on platelets and microparticles was assessed by flow cytometry using PE-CD154 (BD).

Flow cytometry analysis. Flow cytometry was performed on a FACSCanto (BD), and data processed with FlowJo 7.6.5 (Tree Star Inc.). All samples were analyzed using the same voltage settings, and the instrument performance was daily monitored using BD CaliBRITE beads (BD). After correction for nonspecific binding, CD154 and CD62P expression were presented in fluorescence arbitrary units. Microparticles data were expressed in percentage of positive events.

Endothelial and vascular function markers. Polymorphisms G894T and T786C of eNOS protein were analyzed as markers of endothelial function. Genomic DNA was extracted from peripheral blood cells collected in ethylenediaminetetraacetic acid (EDTA) tubes using a PureLink Genomic DNA Mini Kit (Invitrogen). A region containing each polymorphism[32] was amplified by PCR using 1 mg of DNA and 1 mM of specific primers. Amplicons were then digested with specific restriction enzymes, and the digested fragments were visualized in a 2% ethidium bromide agarose gel.

The marker of vascular function chosen in this study was VEGF. Serum concentrations of this growth factor

were measured by specific ELISA assays (designed to measure human VEGF-A) using the Quantikine Human VEGF kit (R&D Systems) according to the manufacturer's protocol. Each sample was measured in duplicate; the intra-assay variation among the duplicates for all samples was <10%. The concentrations of VEGF were expressed in pg/mL.

Statistical analysis. Data were summarized as median and interquartiles 25% and 75% (Q25–Q75) for continuous variables and as proportions for categorical variables. Noncontinuous variables were analyzed using a 2×2 table and Fisher exact test. General linear model ANOVA with Bonferroni correction was used for continuous variables.

In AMI and SA groups, blood markers were measured in the same patient repeatedly in 2 different time points. Therefore, the set of observations are intercorrelated, and appropriate statistical methods were mandatory. A linear mixed effects (LMEs) model was applied. This statistical model describes the longitudinal variations of each patient allowing to estimate differences in average slopes between baseline (day 0) and the other time point, giving a measure of the variation of each blood marker over time. To apply LME, a logarithm transformation was applied to sCD40L, CD40L expression on platelets and microparticles, C-reactive protein, cardiac troponin, N-terminal pro-brain natriuretic peptide, aspartate transaminase, and CK, whereas a square root transformation was applied to VEGF.

The LME model was also used to assess the correlations between sCD40L concentrations and CD40L expression over time and other blood markers, demographic and clinical characteristics, and ongoing therapy.

Linear regression analysis was used to estimate the effect of confounders (risk factors, previous medication, comorbidities, etc) on sCD40L levels for each population and each evaluation point. Each model was crossvalidated with the baseline model.

Values of $P < 0.05$ were considered statistical significant. The calculations were performed using SPSS (version 22.0) and R (version 2.11.1) software.

RESULTS

Soluble concentrations of CD40L. To investigate the sCD40L changes over time in AMI patients, the concentrations were also measured in SA and CTR groups. Soluble CD40L concentrations were reduced in AMI and SA patients at admission compared with CTR (Table II). In AMI and SA patients, sCD40L concentrations increased to 1 month.

The models of the average time changes of sCD40L were highly significant in both AMI ($F = 5.3$; $P = 0.01$) and SA (F = 10.1; $P = 0.003$). Although the sCD40L longitudinal increase was significant in both AMI and SA patients, the time-changes profiles were different ($F = 13.5$; $P = 0.001$), being the rise greater in SA patients that reach values similar to CTR (Table II).

In the 3 groups studied (AMI, SA and CTR), the concentrations of sCD40L measured at admission were not significantly influenced by demographics, risk factors, and comorbidities. In addition, these factors did not modify the average changes of sCD40L over 1 month in AMI and SA groups.

The medication intake is summarized in Table I. The percentage of variability of sCD40L associated to medication at each evaluation point was relatively small in AMI patients and only significant at D30 = 34.8%, $P = 0.02$, being statins the major contributing factor exerting a positive effect on sCD40L levels. This weak influence of medication in sCD40L at discrete time points in AMI group did not reach significance when introduced in the regression model of sCD40L changes over time. Therefore, the average changes of sCD40L through time in AMI and SA patients were not altered by medication intake.

CD40L expression on platelets and microparticles. Platelets continue to be reported as the major source of sCD40L[11,33] in spite previous studies had shown that platelets do not contain enough amount of sCD40L to be responsible for the circulating sCD40L.[34] Recently studies considered sCD40L as a pool of free soluble and microparticle-bound forms.[21] In this perspective, the CD40L expression on platelets and microparticles was assessed.

At hospital admission, the platelet CD40L expression in AMI patients was similar to that in SA and CTR groups (Fig 2). However, the values increased significantly to 1 month on AMI patients ($F = 6.2$; $P = 0.03$; Fig 2).

The percentage of microparticles expressing CD40L was also determined. Although no major changes were verified in AMI comparing to SA and CTR groups, the $CD40L^+$ microparticles were also significantly increased to 1 month after PCI intervention ($F = 6.2$; $P = 0.03$; Fig 1).

Moreover, the expression of CD40L on platelets and on microparticles was not influenced by demographics, risk factors, and comorbidities or medication intake previous to admission.

The association of sCD40L with CD40L expression on platelets and microparticles were also evaluated in the studied groups. Soluble CD40L levels did not correlate with the expression of CD40L neither on platelets ($F < 3.2$; $P > 0.32$ and $r = 0.27$; $P = 0.90$) nor on microparticles ($F < 1.1$; $P > 0.76$ and $r = 0.39$; $P = 0.10$).

Associations of sCD40L to VEGF and eNOS polymorphisms. To clarify the relationship of sCD40L with endothelial dysfunction and angiogenesis in AMI

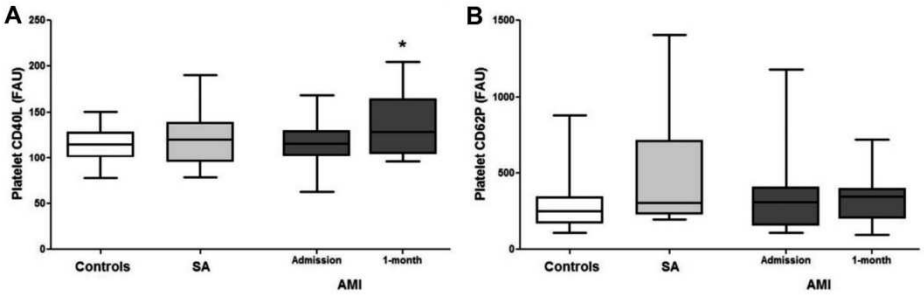

Fig 2. Expression of CD40L (**A**) and CD62P (**B**) on platelets for controls, SA, and AMI patients. *$P < 0.05$ vs AMI group at hospital admission. Plots depicted the dispersion of the numeric values (box, 25%–75% interquartile; horizontal line, median; whiskers, minimum and maximum values). AMI, acute myocardial infarction; SA, stable angina; FAU, fluorescence arbitrary units.

patients, the eNOS polymorphisms and VEGF concentrations were assessed. As AMI and SA patients were monitored twice in 1 month, the LME regression model was used to assess correlations between soluble and bound CD40L, VEGF, and eNOS polymorphisms, whereas a Spearman correlation method was used in CTR group.

Noteworthy associations between sCD40L and endothelial and vascular function markers were obtained for AMI patients.

The G894T polymorphism of eNOS corresponds to a modification of the coding sequence ($Glu^{298} \rightarrow Asp$) which results in the incorporation of aspartate in place of glutamate.[35] The T786C polymorphism results in the replacement of thymine by cytosine.[35]

In our study, the eNOS G894T polymorphism was associated with the longitudinal variations of sCD40L in AMI ($F = 6.9$; $P = 0.01$) but not in SA patients ($F = 0.001$; $P = 0.97$) and in CTR subjects ($r = -0.35$; $P = 0.79$). No significant associations were observed between sCD40L and the eNOS T786C polymorphism in AMI ($F = 0.21$; $P = 0.65$), SA ($F = 0.14$; $P = 0.72$), or CTR groups ($r = -0.11$; $P = 0.96$).

Furthermore, sCD40L levels were correlated with the time-changes VEGF concentrations in AMI ($F = 9.9$; $P = 0.02$) and SA groups ($F = 9.0$; $P = 0.01$). This statistical dependence was not verified in the CTR group, as far as monotonic relationship between paired data is concerned ($r = 0.19$; $P = 0.21$).

The CD40L expression on platelets and on microparticles was not associated with either of the eNOS polymorphisms or of the VEGF concentrations in AMI, SA, or CTR groups.

Association of sCD40L to platelet CD62P expression. This study also intended to investigate the relation between sCD40L levels and markers of platelet activation, such as P-selectin (CD62P) expression.

No significant correlations were verified between sCD40L levels and the expression of CD62P on platelets in AMI, in SA patients ($F < 1.3$; $P > 0.42$) and in CTR group ($r = -0.30$; $P = 0.13$).

DISCUSSION

In the present work, we studied whether sCD40L was related with markers of vascular function and of platelet activation along disease progression in AMI patients. Novel results were obtained highlighting the association between the time changes of sCD40L over 1 month after myocardial infarct onset and markers of endothelial and vascular function (G894T eNOS polymorphism and VEGF concentrations), but not to the platelet CD62P expression.

The time changes of sCD40L in AMI patients, along disease progression, correlate positively with the eNOS G894T polymorphisms, which is associated with endothelial dysfunction. The G894T polymorphism leads to a conservative replacement of glutamate with aspartate causing conformational alterations in the protein, thereby enhancing its susceptibility to proteolytic cleavage in endothelial cells and vascular tissues.[36] Furthermore, this polymorphism has been associated with low plasma NO concentrations and with higher risk of CAD development.[35,37] In our study, the longitudinal variations of sCD40L were correlated with this polymorphism, suggesting a relationship of G894T polymorphism with endothelial dysfunction along disease progression.

Moreover, the time changes of sCD40L also correlate with the variations of VEGF. VEGF is an endothelial cell-specific mitogen that has been reported to promote collateral vessels formation in ischemic cardiac muscle and tissue repair after injury.[38] Circulating levels of

VEGF could affect the outcome of AMI.[20,38,39] We have previously reported depressed serum VEGF concentrations immediately after AMI which increase over 1 year,[20] with a similar trend to that observed in this study for sCD40L. The association between sCD40L and circulating VEGF was also described by Lim et al[40] in CAD patients; however, in this case, the levels of both markers were increased in those patients. In addition, conflicting data were reported in studies using in vitro or animal models. Urbich et al[10] observed a blockage of VEGF-induced angiogenesis by CD40L that could affect endothelial regeneration after plaque disruption.[10] Other authors had shown that sCD40L transcriptionally regulates VEGF expression in endothelial cells, favoring growth and proliferation, and also promoting angiogenesis in mouse.[41] Our results for AMI patients show a concomitant increase in sCD40L and VEGF levels along AMI progression. Therefore, the association of sCD40L with VEGF in AMI pathophysiology may suggest a role of sCD40L in angiogenesis.

The levels of sCD40L did not significantly correlate with the expression of platelet activation markers, such as CD62P. This may suggest that, along the disease progression in AMI patients, the sCD40L is not related to platelet activation or thrombosis.

Furthermore, no association of sCD40L with membrane-bound CD40L was found, which was not unexpected results. In fact, previous studies suggested that a variety of cells might be the source of sCD40L,[42] what justifies the lack of correlation of sCD40L with a specific cell-type marker verified by us.

As far as cross-sectional testing is concerned, we observed that patients at hospital admission, regardless the acute or stable nature of coronary disease, showed remarkably low levels of sCD40L when compared with healthy subjects. After 1 month, the levels of sCD40L of SA patients rose to levels similar to those of CTR, whereas in AMI patients, the increase of sCD40L was also significant, but less marked. The differences between sCD40L concentrations in CAD (AMI and SA) patients at hospital admission and in controls could be related with the disparity in medication intake. Statins and combined antiplatelet therapy had been referred to lower sCD40L concentrations.[43-47] However, in our study, there was no influence of pre- and postevent medication intake in the longitudinal variations of sCD40L levels, suggesting that the differences observed in the rate of sCD40L increase with time in AMI and SA patients were not related to medication intake. It can be hypothesized that low levels of sCD40L in CAD patients at hospital admission may reflect a persistent binding of CD40L. This may implicate a continuous activation of the signaling pathways in which CD40L is involved. The increase of sCD40L in AMI patients after 1 month proved to be slower than that in SA patients, probably reflecting the magnitude of injury and of involved territories.

In the last 2 decades intensive literature has been published reporting higher sCD40L in patients with both stable and unstable CAD (including AMI).[2,7-9,48-50] However, published results are difficult to compare. Time from disease onset to sample collection is often unspecified, which may cause strong deviations on absolute values of measured parameters.[24-26] Nevertheless, we argue that in the present study, rigorous protocols were applied to mitigate sources of error originated from preanalytical and analytical methods.

Although the sCD40L had been implicated in the endothelial dysfunction and angiogenesis,[3,10-14] there were no data available for AMI patients. Our results for AMI patients show a concomitant increase in sCD40L and VEGF levels along AMI progression and temporal variation of cardiac function markers toward stabilization. Therefore, the association of sCD40L with VEGF in AMI pathophysiology may suggest a role of sCD40L in endothelial dysfunction and angiogenesis. Our results show that, regarding AMI pathophysiology, sCD40L cannot be considered just as a marker of platelet activation. In fact, our results corroborate previous in vitro studies that implicate sCD40L in endothelial dysfunction and vascular function, demonstrating for the first time this association in a clinical observational study. The pathophysiologic implications of these findings are very important. A prognostic value for the sCD40L could be hypothesized along the progression of the disease in AMI patients. However, further studies are mandatory to clarify the time evolution of the link between sCD40L and VEGF in patients after AMI. Exploring this connection may support endothelial dysfunction assessment in AMI and eventually contribute to the establishment of a prognostic value to that link.

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Artigo 2

Ligando CD40 solúvel e estratificação de doentes com enfarte agudo do miocárdio com elevação do segmento ST

Stratification of ST-elevation myocardial infarction patients based on soluble CD40L longitudinal changes

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Involvement of soluble CD40 ligand (sCD40L) in thrombosis and inflammation on the context of coronary artery disease is currently being revised. In that perspective, we had studied the association of sCD40L with markers of platelet activation and markers of endothelial and vascular function. On that cohort, a stratification of patients with acute myocardial infarction (AMI) 1 month after percutaneous coronary intervention (PCI) was observed based on concentrations of sCD40L. The study intended to identify the groups of AMI patients with different profiles of sCD40L concentrations and verify how medication, clinical evolution, biochemical data, and markers of regulation of endothelial function at genetic (endothelial nitric oxide synthase polymorphisms) and post-transcriptional levels (circulating microRNAs) affect sCD40L serum levels. Lower quartiles of sCD40L (<2.3 ng/mL) were associated with higher concentrations of N-terminal pro-brain natriuretic peptide (NT-proBNP), high frequency of G894T polymorphism, and altered expression of a set of microRNAs assumed to be involved in the regulation of endothelial and cardiac function and myocardium hypertrophy, relative to patients in sCD40L upper quartiles. A characteristic sCD40L variation pattern in STEMI patients was identified. Low levels of sCD40L 1 month after PCI distinguish STEMI patients with worse prognosis, a compromised cardiac healing, and a persistent endothelial dysfunction, as given by the association between sCD40L, NT-proBNP, G894T polymorphism, and specific profile of miRNA expression. These results suggest sCD40L could have a prognostic value in STEMI patients. (Translational Research 2016;176:95–104)

Abbreviations: AMI = acute myocardial infraction; CAD = coronary artery disease; eNOS = endothelial nitric oxide synthase; miR = microRNA; NT-proBNP = N-terminal pro-brain natriuretic peptide; PCI = percutaneous coronary intervention; sCD40L = soluble CD40 ligand; STEMI = ST segment elevation myocardial infraction

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AT A GLANCE COMMENTARY

Napoleão P, et al.

Background

- In a previous study evaluating the influence of sCD40L in endothelial and vascular function we found that time changes of sCD40L over 1 month after percutaneous coronary intervention (PCI) stratify STEMI patients;
- Influence of medication, clinical evolution, biochemical data, and markers of regulation of endothelial function at genetic and post-transcriptional levels on sCD40L was assessed.

Translational Significance

- For the first time, we found some patients display low sCD40L concentrations over time (low-sCD40L group) and others show an increase of sCD40L levels 1 month after PCI (high-sCD40L group);
- Patients in the low-sCD40L group were associated to high N-terminal pro-brain natriuretic peptide and altered expression of 3 microRNAs involved in regulation of endothelial and cardiac function;
- Low-sCD40L group shows higher frequency of T allele of G894T polymorphism than in the high-sCD40L group;
- Low levels of sCD40L 1 month after PCI distinguish STEMI patients with worse prognosis, a compromised cardiac healing, and a persistent endothelial dysfunction;
- For STEMI patients, a prognostic value for sCD40L could be suggested.

INTRODUCTION

Current opinion suggests a differential role of soluble CD40 ligand (sCD40L) at different stages of coronary artery disease (CAD), contrasting with traditional view of an unvarying function of CD40L/CD40 system interactions in the disease.[1] Cross-sectional studies have shown circulating levels of sCD40L relate to CAD and acute manifestations of the disease, such as, acute myocardial infarction (AMI).[2-6] However, an unequivocal indication of its utility in chronic or acute CAD manifestations has not been obtained so far.

In the context of CAD, several in vitro studies evidenced the relevance of sCD40L in endothelial dysfunction,[7-10] activation of different types of vascular cells,[11] leukocyte trafficking, and homing,[7,10] among other distinctive processes of the atherosclerotic pathology.

In a previous study conducted by us,[5] we have studied association of sCD40L with markers of endothelial and vascular function and of platelet activation, after AMI. On that cohort, evidences were founded of a stratification of patients with ST segment elevation myocardial infarction (STEMI) based on sCD40L concentrations 1 month after percutaneous coronary intervention (PCI). The present study was designed to identify the groups of STEMI patients with different profiles of sCD40L concentrations and verify how medication, clinical evolution, biochemical data, and markers of regulation of endothelial function at genetic (endothelial nitric oxide synthase [eNOS] polymorphisms) and post-transcriptional levels (circulating microRNAs, which are key regulators of cardiac function[12]) affect sCD40L serum levels.

MATERIALS AND METHODS

Study groups. A consecutive sample of 200 subjects (57 women and 143 men) were recruited from the Cardiology Department and from the outpatient clinic of cardiovascular risk in Santa Marta Hospital (Centro Hospitalar de Lisboa Central, Lisbon, Portugal). Among those, 89 STEMI patients (with documented ST-elevation changes, creatine kinase >3 times normal and cardiac troponin >0.01 ng/mL), undergoing PCI as reperfusion therapy, were enrolled during the first 6 hours of onset of chest pain; and 48 age-matched patients with stable angina (SA), presenting typical chest discomfort and/or positive stress tests, which were submitted to coronary angiography. Furthermore, 63 healthy volunteers, the control group (CTR), with negative stress test, absence of any history of coronary disease, life-threatening diseases, or any other disease or condition that impairs compliance, were also recruited. These volunteers were not submitted to coronary angiography.

SA patients and CTR subjects were used as predictors of altered endothelial regulation in AMI.

Exclusion criteria included age >85 years, significant comorbidities as peripheral artery disease or carotid artery disease, known antecedents of malignant, infectious and concurrent inflammatory diseases, chronic renal insufficiency, and previous myocardial infarction in the last 5 years.

Laboratory blood analysis for clinical chemistry, including N-terminal pro-brain natriuretic peptide (NT-proBNP), C-reactive protein (CRP) and cardiac troponin T (cTnT), were measured in all patients and controls (Table I).

Study protocol and follow-up evaluation. STEMI and SA patients were monitored at 3 time points as follows: day 0 before PCI and administration of therapy, such as

Table I. Baseline characteristics of patients and controls, expressed as median and interquartile (Q25–Q75), unless otherwise indicated

	CTR (n = 63)	SA (n = 48)	STEMI (n = 89)
Demographics			
Sex (f/m)	23/40	13/35	21/68
Age (y)	55 (47–65)	63 (57–73)	63 (54–72)
Risk factors/comorbidities*			
Body mass index (kg/m^2)	26 (24–28)	28 (25–29)	27 (24–30)
Waist perimeter (cm)	86 (82–94)	96 (91–102)	99 (89–106)
Smoking, n (%)	4 (6)	4 (8)	39 (44)†‡
Dyslipidemia, n (%)	29 (46)	35 (73)†	47 (53)†
Hypertension, n (%)	18 (29)	37 (77)†	58 (65)†
Diabetes mellitus, n (%)	2 (3)	16 (33)†	35 (39)†
Previous event medication			
No medication, n (%)	46 (73)	5 (10)†	27 (30)†‡
Aspirin, n (%)	4 (6)	32 (67)†	41 (46)†‡
ACE-inhibitor, n (%)	9 (14)	24 (50)†	30 (34)†
β-blockers, n (%)	3 (5)	22 (46)†	26 (29)†‡
Statins, n (%)	10 (16)	37 (77)†	35 (39)†‡
Diagnostic markers			
CRP (mg/dL)	3.1 (1.2–3.5)	3.2 (1.6–5.9)	6.30 (3.2–13)
cTnT (ng/mL)	<0.01§	<0.01§	0.47 (0.07–3.6)††
NT-proBNP (pg/mL)	38 (16–64)	98 (51–247)	356 (145–1577)†‡
eNOS polymorphisms			
G894T, GG/T (%)	38/62	37/63	38/63
T786C, TT/C (%)	43/57	50/50	28/72

Abbreviations: ACE-inhibitors, angiotensin-converting enzyme inhibitor; *BMI,* body mass index; *CRP,* C-reactive protein; *cTnT,* C-reactive protein and cardiac troponin T; *eNOS,* endothelial nitric oxide synthase; *NT-proBNP,* N-terminal pro-brain natriuretic peptide; *SA,* stable angina.
*Smoking: inhaled use of cigarettes, cigars, or pipes in any quantity, in the year previous to admission; dyslipidemia: total serum cholesterol ≥190 mg/dL or serum triglycerides ≥180 mg/dL or use of lipid-lowering medication; hypertension: systolic blood pressure ≥140 mm Hg or diastolic, blood pressure ≥90 mm Hg or use of antihypertensive therapy; diabetes mellitus: fasting plasma glucose concentration ≥7.0 mmol/L or 2 h plasma, glucose ≥11.1 mmol/L or confirmed as clinically known and treated diabetes, mellitus.
†$P < 0.05$ versus CTR group.
‡$P < 0.05$ versus SA group.
§Values below detection limit.

antithrombotic agents and IIb/IIIa inhibitors, and 2 and 30 days after PCI. This protocol was designed to evaluate AMI patients at acute phase of AMI (rupture and coronary occlusion) and at the early recovery phase (cardiac healing and left ventricular remodeling), 2 and 30 days later. All patients were medicated by an assistance clinician. Previous studies using this cohort indicated that influence of medication in the values of inflammatory markers lasted for several days after PCI.[5,13] Therefore, patients' assessment 1 month after PCI represents the period of time for medication and clinical stabilization.

A follow-up was performed by consulting medical records and/or through telephone contact, for a period up to 1 year. Evaluated primary end point was cardiovascular death.

The study was conducted according to principles expressed in the Declaration of Helsinki and was approved by the Ethical Committee Board of Centro Hospitalar de Lisboa Central. All patients and volunteers enrolled signed a written consent following a full explanation of the study.

Blood sampling. Blood samples were drawn into blood collection tubes without additives. For STEMI and SA patients at the hospital, admission blood was collected immediately before PCI, which in case of STEMI patients was performed at hospital admission (within 6 hours of onset of chest pain). For subsequent time points and healthy volunteers (CTR), fasting blood samples were collected early in the morning to avoid possible circadian variations of measured parameters.[14]

Serum was collected after centrifugation (500 *g* for 10 minutes). Aliquots were stored at −80°C until further analysis (no longer than 6 months). Samples were thawed only once.

sCD40L measurement. Concentrations of sCD40L were measured in serum by enzyme-linked immunosorbent assay commercial kit (R&D Systems). Each sample was measured in duplicate, and intra-

assay variation among duplicates for all samples was <10%. Concentrations were expressed in nanogram per milliliter.

Having into consideration our previous exploratory analysis,[5] serum was chosen to determine sCD40L by enzyme-linked immunosorbent assay. In addition, to safeguard sCD40L stability, temperature was kept at 4°C in all steps after blood collection, that is, transport and processing. A rigorous protocol was applied to mitigate source of error originating from pre-analytical and analytical methodologies.[5]

DNA extraction and genotyping. Polymorphisms G894T and T786C of eNOS protein were analyzed. Genomic DNA was extracted from peripheral blood cells collected in EDTA tubes using a PureLink Genomic DNA Mini Kit (Invitrogen). A region containing each polymorphism[15] was amplified by polymerase chain reaction (PCR) using 1 mg of DNA and 1 mM of specific primers. Amplicons were then digested with specific restriction enzymes, and the digested fragments were visualized in a 2% ethidium bromide agarose gel.

G894T polymorphism corresponds to a modification in the coding sequence ($Glu^{298} \rightarrow Asp$) located at the 894th position in exon 7 of the eNOS gene, which results in incorporation of aspartate in place of glutamate.[16] T786C polymorphism result in a replacement of thymine by cytosine at nucleotide-786 position, decreasing transcription of eNOS gene and reducing in half the promoter activity.[15]

MicroRNA quantification analysis. RNA was extracted from serum samples using miRCURY RNA Isolation Kit (Exiqon). Complementary DNA was synthesized from RNA using Universal cDNA synthesis kit (Exiqon).

A microarray screening of circulating microRNAs was performed by quantitative PCR using a panel of 179 microRNAs (miRCURY LNA Universal RT microRNA PCR assays; Exiqon). Based on those results, 19 microRNAs were preselected, namely, miR-1, miR-15b, miR-19b, miR-20b, miR-21, miR-27b, miR-32, miR-99a, miR-125b, miR-126, miR-133a, miR-145, miR-146, miR-147a, miR-155, miR-208b, miR-222, miR-382, and miR-485.

Expression of preselected microRNAs (LNA PCR primer, Exiqon) was assessed. Amplification was performed by quantitative real-time PCR followed by determination of melting curve according to the conditions as follows: 95°C for 10 minutes followed by 45 cycles of 95°C for 10 seconds and 60°C for 60 seconds. Triplicates were performed. Raw Ct values were normalized by global normalization method in each sample, and expression levels of all microRNAs calculated by the Delta-Delta-Ct method as implemented in DataAssist v2 software. Fold changes >2 were considered significant. Results are expressed in relative quantification, which shows fold changes of a specific microRNA in 2 populations.[17] Comparisons were as follows: CTR versus low-sCD40L group; CTR versus high-sCD40L group; low-sCD40L versus high-sCD40L group; CTR versus SA; low-sCD40L group versus SA; and high-sCD40L group versus SA. For STEMI and SA patients, values were compared at hospital admission and 30 days later. A relative quantification value different than 1 indicates different expressions of a specific microRNA in 2 populations.[17]

Statistical analysis. Data were summarized as median and interquartiles (IQ) 25% and 75% (Q25–Q75) for continuous variables and as proportions for categorical variables. Noncontinuous variables were analyzed using a 2×2 table and Fisher exact test and using a general linear model analysis of variance with Bonferroni correction for continuous variables.

As measures of blood markers for each STEMI and SA patient were not independent over time, a linear mixed effects (LME) model was applied. This statistical model describes longitudinal variations of each patient allowing to estimate differences in average slopes between baseline (hospital admission, day 0) and other time points, giving a measure of the variation of each blood marker over time. To apply LME, a logarithm transformation was applied to sCD40L.

LME model was also used to compare longitudinal variation between groups of patients (SA, STEMI and sCD40L-based STEMI groups and SA), and assess correlations between sCD40L concentrations over time and other blood markers, demographics, clinical parameters, and therapy.

Values of $P < 0.05$ were considered statistically significant. Calculations were performed using SPSS (version 22.0) and R (version 2.11.1) software.

RESULTS

STEMI patients' stratification based on sCD40L levels measured 30 days after PCI. Soluble sCD40L concentrations were reduced at baseline in STEMI and SA patients when compared with CTR. One month after PCI, sCD40L levels increased in SA patients to values similar to CTR, whereas in STEMI patients, they remained significantly diminished (Fig 1, *A*). These changes of sCD40L concentrations over time were significant in both STEMI and SA as previously reported by us.[5]

Two groups of patients were identified based on the 50% percentile of sCD40L concentrations measured 1 month after PCI (cutoff concentration of 2.26 ng/mL). The low-sCD40L group describes cases with sCD40L concentrations below the 50% percentile (median; IQ;

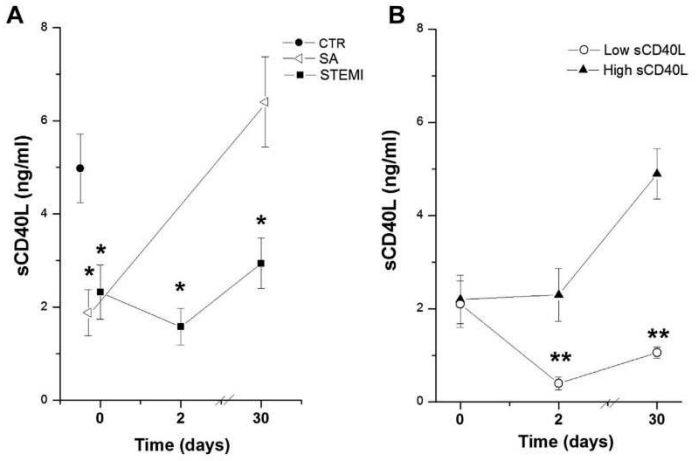

Fig 1. Variations of sCD40L levels over 1 mo after PCI in studied populations (CTR group, SA and STEMI patients, **A**) and in STEMI groups, low- and high-sCD40L, based on sCD40L concentrations measured 1 mo after PCI (**B**). Significances ($P < 0.05$) versus CTR (*) and versus low-sCD40L group (**) in each evaluation time point are indicated. PCI, percutaneous coronary intervention; SA, stable angina.

minimum–maximum: 0.88; 0.55–1.55; 0.36–2.26 ng/mL), whereas the high-sCD40L group describes cases with sCD40L levels above the 50% percentile (median; IQ; minimum–maximum: 3.48; 3.00–5.66; 2.33–11.2 ng/mL). Soluble CD40L concentrations in high-sCD40L group of STEMI patients were similar to sCD40L levels of CTR and of SA patients at day 30.

Modeling sCD40L average changes over time in these 2 groups of STEMI patients, a significant difference in longitudinal profile was observed ($F = 8.54$, $P < 0.0001$) as can be inferred in Fig 1, *B*. Progressive average increase in sCD40L levels from hospital admission until day 30 in the high-sCD40L group contrasts with the average decrease in sCD40L after AMI in the low-sCD40L group.

High- and low-sCD40L groups did not differ in terms of number of patients, demographics, risk factors, and comorbidities (Table II). Influence of medication was addressed previously[5] in this cohort, and no significant correlations between medication intake (pre-event and after hospital discharge) with sCD40L levels in serum were verified. As described in Table II, the 2 groups of STEMI patients revealed similar prescriptions; therefore, medicines do not contribute to stratification.

A 1-year clinical follow-up was considered having cardiovascular death as primary end point. In the overall of STEMI patients assessed, 5 patients died of cardiovascular causes (mean survival time of 4 months). Three of these patients died <1 month after PCI; consequently, they cannot account for sCD40L stratification. However, on those patients, sCD40L concentrations at day 2 (0.77, 0.68–1.02) were similar to those of the low-sCD40L group (Fig 1). Furthermore, the remaining 2 deaths (1 year after PCI) correspond to patients who were classified in the low-sCD40L group.

Longitudinal changes of NT-proBNP differed in sCD40L-based STEMI groups. NT-proBNP showed significant changes in the 2 groups of STEMI patients (Table III). NT-proBNP concentrations 2 and 30 days after PCI significantly differ in both groups ($P < 0.05$). Concentrations of this natriuretic peptide remain high over 30 days in the low-sCD40L group contrasting with those in the high-sCD40L group, where levels sharply decrease to day 30. Consequently, NT-proBNP time-variation trend over 30 days significantly differed in the low- and high-sCD40L groups ($F = 6.54$, $P = 0.015$). In addition, in STEMI patients, sCD40L increases were correlated with decreases in NT-proBNP concentrations.

eNOS genotypes in sCD40L-based STEMI groups. Frequency of polymorphisms of eNOS differed in the 2 sCD40L groups of STEMI patients (Fig 2). Regarding G894T polymorphism, T allele was more frequent in the low-sCD40L group (82%) in comparison with that in the high-sCD40L group (33%, $P < 0.05$). Frequency of T allele in the CTR group was diverse (Table I). No remarkable differences in frequency of eNOS T786C polymorphism were

Table II. Characteristics of low- and high-sCD40L groups of STEMI patients

	Low-sCD40L (n = 26)	High-sCD40L (n = 25)
Sex (f/m)	5/15	2/18
Age (y)	62 (56–75)	56 (48–68)
BMI (kg/m^2)	27 (24–31)	28 (25–31)
Waist perimeter (cm)	98 (82–112)	100 (94–105)
Risk factors/comorbidities		
Smoking	10 (38)	12 (48)
Dyslipidemia	16 (62)	17 (68)
Hypertension	17 (65)	20 (80)
Diabetes mellitus	7 (27)	14 (52)
Medication—pre-event		
No medication, n (%)	19 (73)	19 (76)
Aspirin, n (%)	19 (35)	13 (52)
ACE-inhibitor, n (%)	15 (58)	8 (32)
Anti-platelet inhibitors, n (%)	6 (23)	9 (36)
β-blockers, n (%)	6 (23)	11 (44)
Statins, n (%)	9 (35)	14 (56)
Medication—discharge		
Aspirin, n (%)	22 (85)	25 (100)
ACE-inhibitor, n (%)	18 (69)	22 (88)
Anti-platelet inhibitors, n (%)	124 (92)	25 (100)
β-blockers, n (%)	17 (65)	20 (80)
Statins, n (%)	22 (85)	25 (100)

Abbreviations: ACE-inhibitors, angiotensin-converting enzyme inhibitor; *BMI*, body mass index.
Values are medians and interquartiles (Q25–Q75), unless indicated.

observed in both sCD40L-based STEMI groups and CTR.

MicroRNAs profiles in the sCD40L-based STEMI groups. MicroRNA quantification of 19 preselected microRNAs was performed in all samples of CTR, SA, and STEMI patients at days 0 and 30. Differences were found for miR-19b, miR-145, and miR-222 (Fig 3).

At hospital admission, STEMI patients did not show atypical microRNAs expression profile compared with that of the CTR group. However, 1 month after PCI, patients in sCD40L groups exhibited distinct microRNAs expression. No differences were verified in none of the 19 microRNAs quantified in the high-sCD40L group at day 30 versus CTR or SA. However, in the low-sCD40L group, miR-19b expression was 3-fold higher compared with that in CTR (Fig 3, *A*). Furthermore, at day 30, expression of miR-19b and miR-222 was both 4-fold higher in the low-sCD40L group comparing with SA patients (Fig 3, *A* and *C*).

When low- and high-sCD40L groups were compared, striking differences in microRNA profile involving expression of miR-19b, miR-145, and miR-222 were observed (Fig 3). At day 30, expression of miR-19b, miR-145, and miR-222 in the low-sCD40L group was 4.5–6-fold increased comparing with the high-sCD40L group (Fig 3).

Therefore, the low-sCD40L group was consistently associated with an altered post-transcriptional profile of microRNAs, whereas the high-sCD40L group showed similarities with CTR and SA.

Table III. Concentrations of CRP, cTnT, and NT-proBNP measured at 3 time points (hospital admission—days 0, 2, and 30 after PCI) in low- and high-sCD40L groups of STEMI patients

	CRP (mg/dL)	cTnT (ng/mL)	NT-proBNP (pg/mL)
Low-sCD40L			
Day 0	4.3 (3.2–7.4)	0.85 (0.07–2.9)	356 (158–1506)
Day 2	36 (15–47)	2.7 (1.9–4.0)	1567 (851–3125)
Day 30	3.6 (3.2–7.2)	<0.01*	1090 (625–2257)
High-sCD40L			
Day 0	5.2 (3.2–9.3)	0.24 (0.06–3.5)	175 (80–1063)
Day 2	21 (11–33)	2.2 (1.7–3.1)	628 (369–1423)†
Day 30	3.2 (1.7–5.3)	<0.01*	396 (301–707)†

Abbreviations: CRP, C-reactive protein; *cTnT*, C-reactive protein and cardiac troponin T; *NT-proBNP*, N-terminal pro-brain natriuretic peptide; *PCI*, percutaneous coronary intervention.
Values are median and interquartiles (Q25–Q75).
*Below detection limit.
†Significantly different from the low-sCD40L group, $P < 0.05$.

DISCUSSION

This work showed sCD40L changes after PCI stratify STEMI patients, and these changes were associated with genetic and post-transcriptional markers. Results shown sCD40L concentrations 1 month after PCI stratify patients in 2 groups as follows: (1) a group of patients showing persistently low sCD40L concentrations over time (low-sCD40L group) and (2) a group of patients where sCD40L levels progressively increase to day 30 (high-sCD40L group).

Drug intake and clinical characteristics add no explanation to the stratification of STEMI patients. Nevertheless, in the low-sCD40L group, low sCD40L levels were linked to high concentrations of NT-proBNP, higher frequency of eNOS polymorphism G894T, and altered expression of microRNAs. This profile clearly departs from the high-sCD40L group, which shares similarities with CTR for sCD40L concentrations and microRNAs profile. The high-sCD40L group also showed comparable sCD40L longitudinal profile with SA patients 1 month after PCI.

Stratification of CAD patients based on sCD40L levels was previously proposed.[4,18,19] Increased sCD40L levels have been used to identify patients with unstable CAD or at risk for acute syndromes, independently of other predictive variables.[6,18,20] Apart from important methodology issues previously

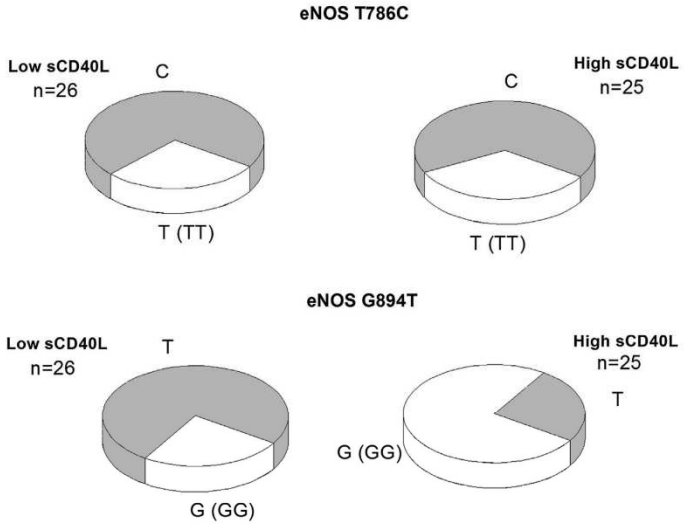

Fig 2. Variations of genotyping polymorphisms of eNOS T786C and G894T in low- and high-sCD40L groups of STEMI patients.

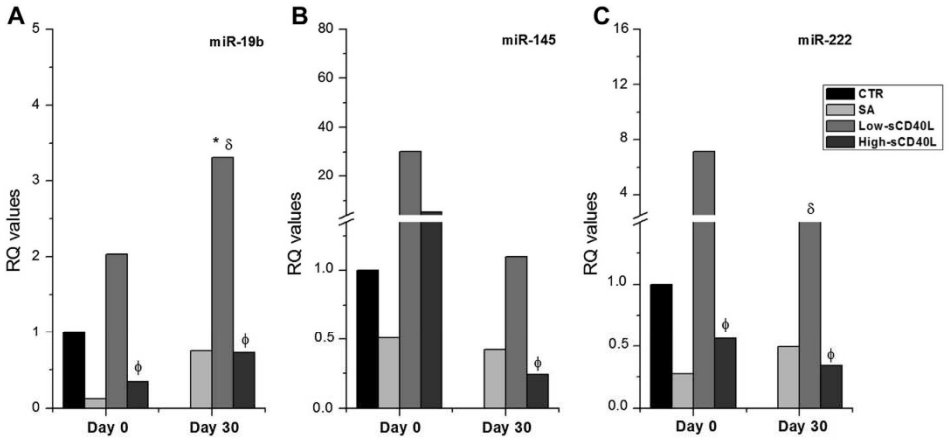

Fig 3. MicroRNAs miR-19b (**A**), miR-145 (**B**) and miR-222 (**C**) serum levels in CTR and SA groups and in sCD40L-based STEMI groups. Values represented are RQ values versus CTR group (reference group). $^{*}P < 0.05$ versus CTR, $^{\delta}P < 0.05$ versus SA group at day 30, $^{\varphi}P < 0.05$ versus low-sCD40L group at day 30. RQ, relative quantification; SA, stable angina.

mentioned by us,[5] none of these studies exclusively assessed STEMI patients or variations of sCD40L over time as in the current work. Furthermore, more recent studies not only fail to demonstrate an association of high level of sCD40L with increased risk of cardiovascular events,[21-24] but in fact, indicate an inverse relation of sCD40L levels with risk of recurrent ischemia.[23]

The CD40/CD40L system represents a major co-stimulator system triggering different signaling pathways mainly attending as amplification of immune

and inflammatory responses,[25] including in endothelial cells.[26,27] Therefore, CD40L can be implicated in promoting inflammation,[9] angiogenesis,[28] and endothelial dysfunction.[7,29] Furthermore, overexpression of CD40 and CD40L was demonstrated in hearts of mice with myocarditis providing a mechanistic link between damaged myocardium and immune response.[30]

Soluble CD40L form also plays a role in CD40/CD40L interaction between immune cells and nonimmune cells in a much wider context of the disease than thrombosis.[5,31] Increases in solubilized CD40L may indicate lesser interaction with membrane-bound CD40 in target cells, either by release of CD40L exposed on cell surface or decreased expression of CD40L due to lack of extracellular and intracellular inhibitory stimulus of integrins and inflammatory molecules,[31] rather than representing an increased costimulatory possibility. In fact, high affinity of sCD40L for CD40 when tumor necrosis factor alpha receptors and integrins are expressed was reported.[32,33] This hypothesis finds an echo in the results obtained in our study, suggesting a detrimental effect of low sCD40L in AMI patients.

To our knowledge, this is the first clinical study directly linking sCD40L changes in the AMI evolution with post-transcriptional microRNAs profile. Previous in vitro and animal studies establish several microRNAs as having post-transcriptional regulator function of several pathways implicated in AMI.[34,35] Among those are some microRNAs identified in the present study as upregulated in the low-sCD40L group versus controls such as miR-145. Previous studies positively related miR-145 to troponin T release establishing involvement of this microRNA in neointima repair in response to vascular injury. Thus, elevated miR-145 levels in the low-sCD40L group might be a result of vessel and myocardial injury as a consequence of MI. The miR-19b and miR-222 had also been related to endothelial and vascular dysfunction.[36,37] MiR-222 has also been associated with vascular wound healing after injury and myocardium function and could contribute to heart diseases such as hypertrophic cardiomyopathy.[37] Taking together, there seems to exist a profile of microRNAs related to endothelial dysfunction, compromised cardiac function, and myocardium hypertrophy associated with the low-sCD40L group, which is clearly different from controls, SA patients, and the high-sCD40L group.

Moreover, the low sCD40L group showed a higher frequency of T allele of G894T eNOS polymorphism. Prevalence of G894T eNOS polymorphism has been associated with physiological alterations and worse clinical outcomes, for example, low plasma NO concentrations and risk of CAD development.[16,38] This polymorphism leads to a conservative replacement of glutamate with aspartate causing conformational alterations.[39] Consequently, eNOS susceptibility to proteolytic cleavage in endothelial cells and vascular tissues is higher, which has a functional effect on the enzyme,[39] and subsequently on NO bioactivity.[39,40] Antoniades et al[40] report that, in humans, G894T polymorphism may modify eNOS activity and is associated with endothelial dysfunction by decreasing NO but only under endothelial cell stimulation. According to those authors, the G894T polymorphism has no effect on the control group.[40] These previous results suggest heterozygosity for the T allele could be associated with a compromised endothelial function in low-sCD40L STEMI patients, but not in the healthy subjects (CTR).

Finally, in our study, STEMI patients in the low-sCD40L group had higher NT-proBNP values, which are associated with adverse outcomes[41-43] and is a clinically relevant marker of left ventricular dysfunction.[44] Therefore, lower sCD40L concentrations can be associated with worse outcome and left ventricular dysfunction, as far as NT-proBNP variations are concerned.

In conclusion, our study shows STEMI patients with lower sCD40L levels have worse prognosis, a compromised myocardium function recovery, and a persistent endothelial dysfunction, as given by relationship between sCD40L, NT-proBNP, specific profile of microRNA expression, and frequency of G894T eNOS polymorphism. Results point out to involvement of sCD40L not only in thrombosis and inflammation but also in other important processes in cardiac, endothelial cells, and vascular system dysfunction, as we reported previously.[5] This hypothesis could explain why the sCD40L-based stratification of STEMI patients was observed 1 month after PCI, in a period when the thrombotic effect is already diminished.

We hypothesize that in STEMI patients low sCD40L levels reflect a compromised recovery capacity of myocardium after infarct and persistent endothelial dysfunction. However, further mechanistic studies are mandatory to understand the molecular pathways involved in sCD40L-associated cardiac healing. Nevertheless, sCD40L could have a prognostic value in STEMI patients as a new biomarker of both endothelial and vascular function, being able to identify patients with higher risk of compromised cardiac healing.

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Conflicts of Interest: All authors signing this article have read the journal's policy on conflicts of interest and have none to declare.

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Artigo 3

Influência de polimorfismos plaquetários na isquemia arterial dos membros inferiores em doentes com diabetes mellitus tipo II

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Influence of genetic variations in platelet glycoproteins and eNOS in the development of arterial ischaemia of lower limbs in type 2 diabetes mellitus patients

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HIGHLIGHTS

- Arterial ischaemia of lower limbs is prevalent in diabetes mellitus.
- Ischaemia genetic predisposition was assessed in type 2 diabetes mellitus.
- Kozak polymorphism in GPIbα was link to a higher risk of developing arterial ischaemia of lower limbs in type 2 diabetes.
- Podiatry may be complemented with laboratory risk markers.

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ABSTRACT

Endothelial and platelet dysfunction increase the atherothrombotic risk in diabetes mellitus patients. Therefore, arterial ischaemia of lower limbs is an important complication in diabetes mellitus. In the present work, type 2 diabetic patients were classified by a podiatrist into presence or absence of arterial ischaemia of lower limbs. Several polymorphisms in platelet glycoproteins and eNOS genes were evaluated. Our results suggest that the −5CC genotype in Kozak sequence of GPIbα may be associated with a higher risk of developing arterial ischaemia of lower limbs in type 2 diabetes mellitus patients.

1. Introduction

According to the International Diabetes Foundation, the prevalence of people living with diabetes in Europe in 2015 was about 59.8 millions. Endothelial dysfunction and accelerated atherosclerosis are important factors in the pathogenesis of diabetes mellitus [1]. Consequently, diabetes is associated with a high risk of atherothrombotic events [2], where platelets play a pivotal role, presenting an hyper-reactive state in diabetic patients [3,4]. Importantly, peripheral arterial disease is a powerful indicator of systemic atherosclerosis [5] and it has a prevalence of 10–40% among patients with diabetes [6,7].

Lower limb disease includes peripheral arterial disease, peripheral neuropathy and foot ulceration, and is twice as common in diabetics compared with no diabetic individuals, affecting 30% of diabetics older than 40 years [8]. Consequently, diabetes mellitus patients have a lifetime risk of developing a foot ulcer of 15% [8].

There is a lack of knowledge about genetic aspects that can contribute to the development of lower limbs complications in diabetes mellitus. Thus, the thrombotic predisposition to arterial ischaemia of the lower limbs in type 2 diabetic patients is assessed by evaluating polymorphisms in the three major platelet membrane adhesion receptors and endothelial nitric oxide synthase (eNOS).

2. Material and methods

The presence of arterial ischaemia of lower limbs was evaluated by a podiatric physician based on specific criteria, such as feet weak pulse or absent and ankle-brachial index (ABI), and non-specific

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Table 1
Genotypic frequency of glycoprotein and eNOS polymorphisms amongst type 2 diabetes mellitus patients with and without arterial ischaemia of lower limbs.

Polymorphisms	Without arterial ischaemia of lower limbs (n < 60)		With arterial ischaemia of lower limbs (n < 29)	
	n	%	n	%
HPA-2				
aa	53	91.4	25	89.3
ab	5	8.6	3	10.7
PI^A				
A1/A1	45	76.3	22	78.6
A1/A2	12	20.3	5	18.5
A2/A2	2	3.4	1	3.7
C807T				
C807C	25	42.4	9	32.1
C807T	28	47.5	14	50.0
T807T	6	10.2	5	17.9
Kozak				
−5TT	42	72.4	24	85.7
−5TC	15	25.9	2	7.1
−5CC	1	1.7	2	7.1
VNTR at *GP1BA*				
BC	4	6.9	1	3.6
CC	44	75.9	22	78.6
CD	9	15.5	5	17.9
DD	1	1.7	0	0
NOS3 T-786C				
TT	19	33.3	9	34.6
TC	26	45.6	12	46.2
CC	12	21.1	5	19.2
NOS3 G894T				
GG	24	41.4	15	55.6
GT	25	43.1	10	37.0
TT	9	15.5	2	7.4

criteria based on foot inspection, such as cold skin, alteration in skin colour and hairiness [9–13]. More precisely, feet pulse was determined by arterial doppler ultrasound with ultrasound gel. The doppler sonography at the ankle was used to determine the systolic blood pressure in tibial posterior artery and dorsalis pedis artery. The blood pressure cuff and the stethoscope were used to measure the systolic blood pressure in brachial artery. The ABI was calculated by dividing the highest pressure in ankle by the systolic pressure at the arm. An ABI ≤0.9 suggests peripheral ischaemia, as defined by practice guidelines [14,15].

In this study, 87 type 2 diabetes mellitus patients were enrolled and characterized by presence or absence of arterial ischaemia of lower limbs. All donors gave written informed consent to the protocol, which was approved by the local ethics committee. Genomic DNA extraction (PureLink™ Genomic DNA Mini Kit, Invitrogen) was obtained by blood samples and polymorphisms were evaluated by PCR-RFLP, as previously described [16]. The following primers and restriction enzymes were used for the eNOS polymorphisms: forward primer GTGTACCCCACCTGCATTCT, reverse primer CCCAGCAAGGATGTAGTGAC and *HaeIII* (NetLabs) restriction enzyme were used for T-786C polymorphism; forward primer AGCCTCGGTGAGATAAAGGA, reverse primer CCAATTTCCAGCAGCATGT and *MboI* (Fermentas) restriction enzyme were used for G894T polymorphism.

Platelet glycoprotein (GP) polymorphisms, namely −5T/C in the Kozak sequence at *GP1BA* gene (GPIb), C807T at *ITGA2* gene (GPIa), HPA-2 at *GP1BA* gene (GPIb), PI^A at *ITGB3* gene (GPIIIa), a variable number of tandem repeats (VNTR) at the *GP1BA* gene (GPIb), and the *NOS3* (eNOS) gene polymorphisms T-786C and G894T, were evaluated.

The genotype and allelic frequencies of polymorphisms were determined in individuals with type 2 diabetes mellitus with or without arterial ischaemia of lower limbs. The Hardy–Weinberg equilibrium was tested by means of a chi-square test using observed versus expected genotypic frequencies for a significance level of 0.05. Binary logistic regression was used to determine the association between arterial ischaemia of lower limbs and polymorphisms. IBM SPSS Statistics v.22 was used to perform all the statistical analysis. Differences were significant when $p < 0.05$.

3. Results

Among the donors with type 2 diabetes mellitus, 32.2% (n = 28, 60.7% males, average age of 67 ± 10 years, ranging from 47 to 85 years) presented arterial ischaemia of lower limbs and, 67.8% (n = 59, 44.1% males, average age of 65 ± 12 years, ranging from 27

Table 2
Allelic frequency of glycoprotein and eNOS polymorphisms amongst type 2 diabetes mellitus patients with and without arterial ischaemia of lower limbs.

Polymorphisms	Without arterial ischaemia of lower limbs	With arterial ischaemia of lower limbs
HPA-2		
a	0.96	0.95
b	0.04	0.05
PI^A		
A1	0.86	0.88
A2	0.14	0.12
C807T		
C	0.85	0.57
T	0.15	0.43
Kozak		
C	0.66	0.89
T	0.34	0.11
VNTR at *GP1BA*		
B	0.034	0.02
C	0.871	0.89
D	0.095	0.09
NOS3 T-786C		
T	0.56	0.58
C	0.44	0.42
NOS3 G894T		
G	0.63	0.74
T	0.37	0.26

to 83 years) did not show podiatric evidences of arterial ischaemia of lower limbs. The incidence of previous thrombotic events was similar in patients with (53.57%) and without (50.85%) arterial ischaemia of lower limbs.

Genotypic (Table 1) and allelic (Table 2) frequencies were determined for all tested polymorphisms in type 2 diabetes mellitus patients with and without arterial ischaemia of lower limbs. The Kozak polymorphism in type 2 diabetes mellitus patients with arterial ischaemia of lower limbs was not in Hardy–Weinberg equilibrium, meaning that there are significant differences between observed and expected genotypic frequencies. Only the genotype CC in Kozak polymorphism ($\chi^2_{\text{Wald}} = 4.869$, $p = 0.027$) can predict arterial ischaemia of lower limbs in type 2 diabetes mellitus patients. Moreover, in comparison to TT genotype, type 2 diabetes mellitus patients with the CC genotype presented an increased risk of developing arterial ischaemia of lower limbs (odds ratio = 55.913, 95% confidence interval = [1.568–1994.028]).

4. Discussion

Platelet hyper-reactivity in diabetes contributes to the enhanced atherothrombotic risk [17]. Diabetic state is associated with an increased pro-thrombotic tendency, where platelets show an increased adhesion and aggregation activity [8,3]. This state is an important pre-condition factor of mortality and morbidity in diabetes. Here, we hypothesized if polymorphisms in genes codifying for platelet glycoproteins and eNOS would influence the development of arterial ischaemia of lower limbs in type 2 diabetes mellitus patients. Amongst the studied polymorphisms, it was observed a significant association between the −5T/C polymorphism in the Kozak sequence of GPIbα and the presence of arterial ischaemia of lower limbs in type 2 diabetes mellitus patients. The frequency of the genotype −5CC in the Kozak sequence was found to be 0.4% in the Portuguese population [16]. Nevertheless, type 2 diabetes mellitus patients with arterial ischaemia of lower limbs presented 7.1% of −5CC genotype. Based on literature, this polymorphism seems to have platelet function implications and a role in arterial ischaemia, since the presence of −5C allele increases the surface expression of GPIb-V-IX complex, which may affect the adhesiveness of the platelets [18].

The encouraging preliminary results show that the −5T/C polymorphism in the Kozak sequence of GPIbα might contribute to an increased risk of developing arterial ischaemia of lower limbs in type 2 diabetes. Although atherothrombotic disease is a multifactorial process that involves a complex interaction of environmental and genetic factors, the results from this study provide evidence that −5T/C polymorphism in the Kozak sequence could be a useful tool for podiatrist and endocrinologist to be more aware of minimal signs of ischaemia in these patients.

Conflict of interest

The authors declare that they have no conflict of interests.

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